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UNIVERSITY OF ALBERTA

EFFECTS OF BLOOD BREAKDOWN PRODUCTS ON ISOLATED CEREBROVASCULAR SMOOTH-MUSCLE CELLS

BY

GORDANA MALJKOVIC

A thesis submitted to the Faculty of Graduate Studies and Research in
partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE.

IN

EXPERIMENTAL SURGERY

DEPARTMENT OF SURGERY

EDMONTON, ALBERTA

SPRING 1991

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ISBN 0-315-66682-X

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DEGREE: M.Sc.

YEAR THIS DEGREE GRANTED: 1991

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22nd April, 1991

To my son Marko, with love

ABSTRACT

Delayed cerebral vasospasm following subarachnoid hemorrhage due to the rupture of intracranial aneurysm is fatal or severely disabling in about 70% of patients. However, both its pathogenesis and optimal treatment remain obscure.

An experimental model system was developed to study the effects of putative spasmogens on single smooth-muscle cells isolated from rat basilar artery, using the whole-cell patch-electrode voltage-clamp technique. Cell viability was monitored by fluorescein diacetate uptake.

Single smooth-muscle cells from rat basilar artery were isolated by enzymatic dissociation. The cells contracted in response to agonists such as caffeine (10^{-2} M) and angiotensin II (10^{-7} M), demonstrating that the cells were undamaged by the isolation procedure. Oxyhemoglobin was prepared from rat hemoglobin by reduction with sodium dithionite, and methemoglobin by oxidation with potassium ferricyanide; these agents were subsequently removed by dialysis. Bilirubin was dissolved in dimethyl sulfoxide (DMSO) and used in 1 hour.

Exposure of cerebrovascular smooth-muscle cells to oxyhemoglobin (5×10^{-6} M) caused cell contraction, membrane blebbing, increase in calcium-activated potassium currents, and an irreversible decline in membrane resistance within 5 minutes. By contrast, oxyhemoglobin had no effects on murine neuroblastoma cells in culture. Methemoglobin (10^{-4} M) did not change electrical properties of smooth-muscle cells. DMSO (0.5%) or catalase (300 units/ml) protected cells against the effects of oxyhemoglobin; superoxide dismutase (100-1,000 units/ml) was only partly protective. Exposure of the cells to hydrogen peroxide (5×10^{-4} M) or to superoxide anions generated by xanthine (1 mM) plus xanthine oxidase (10 units/liter) increased calcium-activated potassium currents but did not induce a decline in membrane resistance. Generation of hydroxyl radicals by metal ions plus hydrogen peroxide caused the same effects as oxyhemoglobin. Cells exposed to saturated solutions of bilirubin for up to 11 hours showed only a small degree of contraction, not significantly different from that of control cells treated similarly.

It is concluded that oxyhemoglobin exerts its effects on cerebrovascular smooth-muscle cells by generating free radicals, especially hydroxyl radicals. A role for bilirubin in cerebral vasospasm is not entirely ruled out by these findings.

PREFACE

This thesis reports two studies on single smooth-muscle cells isolated from the rat basilar artery under phase contrast optics with the whole-cell patch-clamp technique. This experimental model system was developed in our laboratory to study the effects of spasmogens that could play a role in the pathogenesis of cerebral vasospasm following subarachnoid hemorrhage.

The introductory chapter of this work deals with our current understanding of cerebral vasospasm following subarachnoid hemorrhage. Specifically, this includes a very brief historical review of cerebral aneurysm and subarachnoid hemorrhage, with clinical diagnosis and the development of therapy. It is followed by our present knowledge of the morphology and pathology of cerebral arteries and aneurysms. A major part of this chapter is devoted to various concepts of the pathogenesis of cerebral arterial spasm and its prevention and treatment. The second part of the introductory chapter deals mainly with our work, including isolation of smooth-muscle cells from cerebral arteries, patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches, and strategies to improve our understanding of cerebral vasospasm at the level of the cell membrane and for evaluating rational therapies for this devastating condition.

Chapter II details a study of the effects of hemoglobin on electrical properties of single isolated smooth-muscle cells from rat basilar artery, with emphasis on those mechanisms of action of oxyhemoglobin on the cerebrovascular smooth muscle that may lead to a therapy for late cerebral vasospasm.

In chapter III is an outline of the effect of bilirubin, another potential spasmogen on electrical properties of cerebrovascular smooth muscle cells. In this study, the effects of bilirubin and oxyhemoglobin are also compared.

Finally, chapter IV summarizes the results of our studies and attempts an outline of directions for future research.

ACKNOWLEDGEMENTS

This thesis could never have been done without the valuable, consistent, and dedicated support of a very special group of individuals.

I wish to thank my supervisor, Dr Norman Stockbridge, for the opportunity he gave me to work in his laboratory, his encouragement, advice, timely guidance, valued criticism and participation during the entire extent of this work.

I am also grateful to the chairman of the Department of Surgery, Dr Bryce Weir, for his benevolent guidance, participation, advice and helpful criticism, as well as financial support of this study.

I wish to express my great appreciation to the members of my supervisory committee, Dr Jim Russell and Dr David Cook, for their invaluable assistance and great guidance at various stages of this project's development.

I thank Dr Joy A. Steele for her patience in teaching me how to use the patch-clamp techniques for high-resolution current recording from cells, as well as for her guidance, advice, and constructive criticism of this project.

I also take this opportunity to express my great appreciation to Ms Ursula Matthews for her unparalleled editing skills and great patience, and for encouragement, and help when I needed it.

A very special thank you note goes to my mother Mileva and my son Marko without whose patience, tolerance, moral support and love nothing would have taken place. I am very grateful to my cat Bronween, for his companionship during many working nights, when it seemed he understood that his quiet presence was giving me a burst of energy.

I thank Drs L.W. Brox, J.F. Henderson, V. Manickavel, J. Przysiezniak, R. Smith, A.N. Spencer, and K. Wong for assistance in technical procedures and the loan of equipment, as well as Drs D. Schiff and E. Karpinski for providing the neuroblastoma cells.

My final thanks are to God, who always gives me strength and inspiration.

This study was supported by grants from the Canadian Heart and Stroke Foundation (to Dr Stockbridge) and by the National Institutes of Health R01 NS25957-01 (to Dr Weir).

TABLE OF CONTENTS

CHAPTER I.

INTRODUCTION 1

Historical review of cerebral aneurysms, subarachnoid hemorrhage and cerebral vasospasm 1

Diagnosis 1

Therapy 2

Aneurysmal subarachnoid hemorrhage: incidence, prevalence, morbidity and mortality 4

Cerebral vasospasm - Part I 9

A. Cerebral arteries and aneurysms 9

Morphology 9

Predisposing factors 10

Intravascular hemodynamics 10

B. Pathology of cerebral vasospasm 12

C. Pathogenesis of cerebral vasospasm 13

1. Hypothesis of mechanical cause 13

2. Hypothesis of catecholamines and neurogenic action 14

3. Hypothesis of myogenic action 15

4. Hypothesis of the action of biogenic amines 15

5. Prostaglandins and leukotrienes 16

6. Blood and blood products 18

D. Clinical management of cerebral vasospasm 20

Cerebral vasospasm - Part II 22

A. Rationale for present approach 22

B. Patch Clamping: Whole-cell and single-channel currents 24

C. Previous studies of electrophysiology 26

Voltage-dependent channels 26

Receptor-operated channels 27

Mechanically sensitive channels 27

D. Objectives 29

Bibliography 30

CHAPTER II.

FREE RADICALS MEDIATE ACTIONS OF OXYHEMOGLOBIN ON CEREBROVASCULAR SMOOTH-MUSCLE CELLS 59
  Introduction 59
  Materials and methods 61
  Results 64
  Discussion 66
  Tables and figures 71
  Bibliography 77

CHAPTER III.

EFFECT OF BILIRUBIN ON ELECTRICAL PROPERTIES OF CELLS 81
  Introduction 81
  Materials and methods 82
  Results 83
  Discussion 86
  Tables and figures 89
  Bibliography 97

CHAPTER IV.

DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS 100
  Discussion 100
  Conclusions 107
  Recommendations 108
  Bibliography 111

APPENDIX I 114
  A. Isolation of smooth-muscle cells 114
  B. Screening assays 115

Cell viability 115
Morphometric analysis 115

APPENDIX II. 116

Characterization of rat basilar artery smooth-muscle cells and their ionic currents 116
Calcium currents 116
Potassium currents 117

Bibliography 119

LIST OF TABLES

Table II-1. Summary of electrophysiological effects of blood breakdown products and free radical generators and scavengers on smooth muscle cells 71

Table II-2. Viability of isolated smooth-muscle cells exposed to blood breakdown products and free radical generators and scavengers 73

LIST OF FIGURES

Figure II-1. Action of oxyhemoglobin on isolated cerebrovascular smooth-muscle cells 74

Figure II-2. Current-voltage plot and current records for an N1E-115 mouse neuroblastoma cell exposed to oxyhemoglobin 75

Figure III-1. The effect of brief exposure to bilirubin on the electrophysiological properties of smooth-muscle cells 89

Figure III-2. Smooth-muscle cell under whole-cell patch-clamp conditions in the control medium 90

Figure III-3. Smooth-muscle cell exposed to bilirubin while under whole-cell patch-clamp 91

Figure III-4. Whole-cell patch-clamp recordings of smooth-muscle cells preincubated in the normal bath solution or bilirubin for up to 11 h 93

Figure III-5. Contraction of smooth-muscle cells in the normal bath solution or bilirubin 94

Figure III-6. Smooth-muscle cell exposed to oxyhemoglobin (whole-cell recordings) 95

LIST OF SYMBOLS AND ABBREVIATIONS

5-HT	5-hydroxytryptamine
AMP	adenosine monophosphate
CNS	central nervous system
CSF	cerebrospinal fluid
GMP	guanosine monophosphate
LT	leukotriene
metHb	methemoglobin
oxyHb	oxyhemoglobin
PG	prostaglandin
SAH	subarachnoid hemorrhage
SOD	superoxide dismutase
TX	thromboxane

CHAPTER I.
INTRODUCTION

HISTORICAL REVIEW OF CEREBRAL ANEURYSMS, SUBARACHNOID HEMORRHAGE AND CEREBRAL VASOSPASM

THE PAST

About 550 BC, an unknown writer, probably a prophet in Babylon during the exile, wrote the Books of the Kings of the Old Testament. In II. KINGS, ch. 4, 18-20 is the first written description of a condition that appears to be subarachnoid hemorrhage: *"And when the child was grown, it fell on a day, that he went out to his father to the reapers. And he said into his father, My head, my head. And he said to a lad, Carry him to his mother. And when he had taken him, and brought him to his mother, he sat on her knees till noon, and then died."* (The Paragraph Bible Annotated, II. KINGS 4, 2-27).

The medical genius, Hippocrates, who was born sometime around 420 BC and died between 377 and 359 BC, appears to have been the first person to record treatment of subarachnoid hemorrhage. He wrote: "*When a person is pained in the back of the head, he is benefited by having the straight vein in the forehead opened.*" (1).

DIAGNOSIS

Almost 20 centuries later, in 1583, Morgagni of Padua wrote about dilation of the cerebral vessels at autopsy (2). In 1778 Biumi of Milan described precisely a ruptured intracranial aneurysm of the carotid artery at autopsy, as well as a clinical account of the disease (2), and in 1813 Blackall described in detail the symptoms and signs of intracranial hemorrhage (2). The first accurate clinical diagnosis of intracranial aneurysm was by Hutchinson in 1864, and confirmed by him in 1875 when his patient died from a pelvic abscess: an aneurysm within an inner part of the middle fossa was verified at autopsy (3). In 1891, Quinke introduced lumbar puncture. There were few advances of note until 1927, when Moniz invented cerebral angiography, the most useful tool in establishing the diagnosis of cerebral aneurysm, and in 1936 Loman and Myerson developed percutaneous carotid angiography (2).

Cerebral computed tomography was introduced by G. N. Hounsfield in 1972. This revolutionary approach to diagnosis of intracranial pathology brought him the Nobel Prize in Medicine in 1979, which he shared with A. M. Cormack, a physicist, who had described the mathematics that could be applied to tomography (4).

THERAPY

Conservative therapy consisting mainly of bed-rest was the only treatment of subarachnoid hemorrhage until the nineteenth century. In addition to bed-rest, Morgagni prescribed ergotamine, potassium iodide, strychnine and galvanic current. Vanzetti (1858) applied prolonged digital compression of the ipsilateral cervical carotid artery in an attempt to cure a carotid aneurysm located in the cavernous sinus (2,3).

One century later, in 1967, Gibbs used antifibrinolytic therapy to reduce the rate of rebleeding by preventing lysis of the clot surrounding a ruptured aneurysm. Others followed him, and early rebleeding was reduced by 50% (3).

The first successful surgical treatment of cerebral aneurysm was done in 1902, by Sir Victor Horsley, who performed unilateral occlusion of the common carotid artery. He was followed by Grey Turner, who in 1926 ligated the internal carotid artery (3).

Early attempts at intracranial surgical treatment of aneurysms were disastrous.

Zeller (1911) and Cushing (1917) attempted intracranial and cervical occlusion of the internal carotid artery; but all three patients died (2,3).

Intracranial approach to surgical treatment of aneurysms have been developed in the past six decades. In 1931 Dott successfully wrapped a muscle around a carotid bifurcation aneurysm (5); those who have followed include Walter Dandy, who in 1937 introduced clip-occlusion of the neck of aneurysms (6).

Great advances were made in neurosurgery and new techniques were developed during the last few years of World War II and in the next decade. This was coincident with the recognition of late cerebral vasospasm as a complication of subarachnoid hemorrhage, manifested by delayed neurologic deterioration, confusion, stupor, coma and sometimes death in patients who were doing well for hours or days after the initial episode. The severity of arterial narrowing was found to parallel this delayed neurologic deterioration and could be monitored with cerebral angiography. In 1951 Ecker and Riemenschneider published the first description of diffuse vasospasm in cerebral arteries after subarachnoid hemorrhage (7).

The clinical grading of patients with subarachnoid bleeding was introduced by Botterel et al. (8) in 1956 and modified by Hunt and Hess (9) in 1968. These studies showed the correlation between the initial neurologic status and the clinical outcome.

The introduction of the operating microscope in the 1960s was one of the greatest technical advances in vascular neurosurgery. For the first time excellent illumination and magnification of the operative field was achieved, improving intracranial operative procedures on aneurysms (10). Since then, microsurgical techniques for cerebral aneurysms have been developed, surgical skills have been sharpened, and anesthesia has greatly improved significantly decreasing the mortality rate during and after operation (11,12).

ANEURYSMAL SUBARACHNOID HEMORRHAGE:

INCIDENCE, PREVALENCE, MORBIDITY AND MORTALITY

Aneurysmal subarachnoidal hemorrhage (SAH) is the commonest cause of cerebral vasospasm. Intracranial aneurysms are classified as saccular (berry), traumatic (dissecting), atherosclerotic and inflammatory. Saccular aneurysm is the commonest of all cerebral aneurysms, and it has been estimated that the incidence in the general population is at least 2% (13-15). Spontaneous rupture of a saccular aneurysm accounts for about 50% of nontraumatic SAH (15-16).

The reported incidence of aneurysmal SAH varies considerably, the number of new cases per 100,000 population per year being highest in Japan with 25 new cases, and lowest in Rhodesia (Zimbabwe) with 3.5 new cases (17). It has been estimated that about 5 million persons in North America (Canada and the USA) harbor saccular cerebral aneurysms and that each year approximately 3,000 aneurysms will rupture in Canada and around 28,000 in the USA; i.e., an incidence of SAH of 10 to 12/100,000 population/year (18-22), the majority of deaths of those with aneurysms being due to other causes.

Aneurysmal SAH is highly lethal: 8-15% of patients die before or soon after coming to medical attention, and the mortality rate increases to 24% in the first 48 hours and to 40-50% by day 14 (20,21,23-26). Of the 50-60% of patients who survive the acute stage, symptomatic cerebral vasospasm may develop in over 25% (27,28). In over 10% of these cases neurologic deterioration and even death will occur 1-2 weeks after the hemorrhage (22,29). Therefore, in patients who survive their first bleeding, the commonest cause of death is not the immediate neurologic damage caused by the loss of the blood flow to the region of brain supplied by the ruptured vessel; instead, it is delayed spasm of other blood vessels, developing 5 days or later, in response to the irritating effects of blood bathing their exterior (30). Besides cerebral ischemia, the complications of rebleeding and hydrocephalus are responsible for the rising mortality rate after the acute episode. Without proper therapy, hydrocephalus may be lethal by a month post-SAH; and rebleeding, if left untreated, could kill up to 1/3 of patients within 10 years of their first stroke. The predominant complication - cerebral vasospasm, known as cerebrovascular spasm or cerebral arterial spasm - can be so severe that brain tissue is destroyed by the lack of oxygen and other blood-borne nutrients. In patients who survive this, the vasospasm reverses spontaneously 7-21 days after the initial event, leaving a spectrum of ischemic neurologic deficits (31-34). Reasons for the presence or absence of cerebral vasospasm are still unknown. The ultimate objective of research on cerebral vasospasm is to reduce disability and death associated with cerebrovascular accidents, or stroke.

Most victims of subarachnoid hemorrhage are in their early 50s and are otherwise healthy. The site of intracranial saccular aneurysms is most commonly in the anterior or middle cerebral artery or the communicating arteries (35). There are isolated reports of vasospasm associated with unruptured aneurysm (36-38), and only one report of intracranial hemorrhage leading to vasospasm of extracerebral external carotid arteries (39).

Numerous studies have been performed to determine the time course of cerebral vasospasm. It is believed there is a transient, but marked, vasoconstriction following introduction of blood into the subarachnoid space of most animals. Reviewing 627 angiograms performed on 293 patients with subarachnoid hemorrhage, Weir et al. detected no cerebral vasoconstriction on angiograms taken within 24 hours (40); and

Wilkins, who reviewed 32 cases of aneurysm that ruptured during angiography, concluded that there was no evidence of acute vasospasm in man (41). The existence of this so-called 'early vasospasm' would be important in terms of experimental work but would have minimal clinical significance even if found in man. On the other hand, it is clear that clinically significant vasospasm, so-called 'late vasospasm', does not become apparent until day 3 and persists for at least a week (40-42).

Cerebral vasospasm is detectable on 30-70% of angiograms performed 4-12 days after the onset of subarachnoid hemorrhage, but attempts to quantify vasospasm reliably have been frustrating; at present, the most useful methods are those developed by Fisher et al. (43) and Weir et al. (40). The time courses of clinical and angiographic vasospasm are parallel, with arterial narrowing developing in up to 70% of patients and delayed ischemic neurologic deficits in only 20-30% (44-49).

Aneurysmal subarachnoidal hemorrhage is the commonest cause of cerebral vasospasm. Ruptured arteriovenous malformation, head injury, and infecion of the central nervous system, especially if associated with large-volume subarachnoid bleeding or inflammation, could mediate cerebral vasospasm (50,51). The occurrence of vasospasm seems to relate directly to the amount or thickness of blood clot in the subarachnoid space (52-55).

Focal neurologic deficits associated with the spastic vessel(s) are characteristic of cerebral vasospasm (46). This intense narrowing of blood vessels can be so severe that brain tissue is destroyed through the development of widespread cerebral ischemia, secondary intracranial hypertension, and brain herniation (43,56,57).

Currently, the diagnosis of cerebral vasospasm is based primarily on the time of onset of neurologic deficits after the hemorrhage, the nature and rate of development of the deficits, and the exclusion of other causes such as rebleeding, intracerebral hematoma, angiographic or surgical complications, and metabolic abnormalities. Angiographic confirmation of arterial constriction is not essential in view of the risks of this invasive procedure, the potential hazard of moving the patient, and the cost (58,59). Further, angiographic visualization of vasospasm does not exclude other causes, as the neurologic deficits are usually multifactorial. Transcranial pulsed doppler ultrasound is an alternative to angiography for establishing a diagnosis of vasospasm (60-64): it is an easily performed, repeatable, and - most important of all - a safe method that may replace neuroangiography for the diagnosis of cerebral arterial narrowing.

The etiology of cerebral vasospasm and neurologic deterioration in patients who have suffered a subarachnoid hemorrhage is complex and unknown. Many hypotheses have been advanced to explain the cause and mechanism of action of this seemingly straightforward complication of the cerebral arterial narrowing, but none has been widely accepted. Problems of applying many of these studies to late cerebral vasospasm in humans include the following:

First, there is no convenient animal model of so-called 'late vasospasm'. This condition has been produced in vivo in monkeys, baboons, dogs, cats, rats, pigs and other species (65-78), usually by the injection of fresh blood into the subarachnoid space, and followed up angiographically. It seems quite probable that the mechanism of development of cerebral vasospasm in most species studied, especially primates, is similar to that in man. The complexity of dealing with intact animals, the hazards of serial angiograms, and cost, have led many researchers to work on isolated cerebral arterial segments. This is a convenient approach but does not provide a model for late vasospasm: it is a model for early vasospasm, which is not clinically important and probably develops differently, and has clinical importance only in the testing of potential agonists as therapy for late vasospasm. Second, the use of different preparations (perfused vessels, rings, etc.), different animal species, and different experimental conditions make the evaluation of cerebral vasospasm in man very difficult. Third, if vasospasm has a delayed onset and a long time course, the vasospastic agent(s) liberated some time after subarachnoid hemorrhage have very prolonged action or they are liberated continuously for many days, and act on receptor system(s) that have no desensitization or autoregulation.

In the first part of this paper, various hypotheses of cerebral vasospasm are discussed, together with studies of cerebral arterial ultrastructure and clinical management of stroke. In the second part the overall objectives of the research to identify the mechanism of cerebral vasospasm at the cellular level are outlined, together with hypotheses to be tested, methods to be used, and a review of knowledge to date.

CEREBRAL VASOSPASM

PART I

A. CEREBRAL ARTERIES AND ANEURYSMS

MORPHOLOGY

The extracerebral intracranial arteries are composed of the three coaxial layers: on the outside collagenous tunica adventitia, in the middle tunica media or muscularis, and tunica intima on the inside. The tunica intima is lined by a layer of endothelial cells. An internal elastic lamina separates the intima from the media. The tunica media is composed of smooth-muscle cells and elastin fibres. In direct contact with the tunica media is the tunica adventitia; the external elastic lamina is absent in the extracerebral intracranial arteries, in contrast to the morphology of extracranial arteries (13). The tunica muscularis may stop abruptly in some places, leaving weak points in the intracranial arterial walls. Some of these defects may be present at birth, but most of them develop postnatally (79). The reason for the existence of these defects is unknown. Are they part of a physiologic mechanism to maintain blood flow during vasoconstriction? Most probably they are not, because of their inconsistent distribution. However, they may act as a raphe between tunica muscularis of the two adjoining arteries, which may constrict in different directions (79). Another possible weak point of the extracerebral intracranial arteries is the internal elastic lamina. It is composed of two layers; the outer layer is a solid mass adjacent to the tunica media, and the luminal surface is a fibrous mesh. At the apices of some bifurcations there are enlarged fenestrations of this fibrous mesh that might contribute to the initiation of microaneurysms with age (80). Berry aneurysms are devoid of tunica media, have absent or damaged internal elastic lamina, and mostly occur at the apices of a major arterial bifurcation, especially on the anterior part of the circle of Willis (14). Multiple aneurysms could be clinically diagnosable in at least 15% of patients (81).

PREDISPOSING FACTORS

Essential hypertension is the leading medical condition in patients with ruptured intracranial aneurysms (82). Increased systolic and diastolic pressures produce higher hemodynamic stress and accelerate formation of atherosclerosis that in turn could contribute to the development and rupture of berry aneurysms (13). There is also a higher chance of development of multiple aneurysms in hypertensive patients (83).

Single and multiple saccular aneurysms are commoner in women than in men. They are rare in childhood and the incidence increases with age, reaching its maximum in the sixth decade in life (16-17). There are some data that indicate autosomal dominant inheritance may play an important role in the development of saccular aneurysms (84,85).

Absence of the internal carotid artery (86) or cerebral arteriovenous malformation (87) results in abnormal hemodynamics that in turn could contribute to the development of cerebral aneurysms (87). Several other disorders, especially those affecting connective tissue, such as polycystic renal disease (88) or giant-cell arteritis (89), may contribute in the development of cerebral arterial aneurysms.

INTRAVASCULAR HEMODYNAMICS

Increased hemodynamic stress at the branching site on the parent artery could induce saccular aneurysm, which would balloon in the direction of flow in the parent artery (90). Turbulence at a cerebral arterial bifurcation can stimulate growth of an aneurysmal sac, by weakening its walls (91). Most aneurysms, especially pulsating ones, rupture suddenly; the others enlarge and thrombose (13,92).

Bleeding from a ruptured aneurysm is usually sudden and dramatic; but this is short-lived, because of the contraction of the smooth-muscle cells and the ability of the cerebrospinal fluid (CSF) to speed up the formation of clots. In the first 2 weeks this protective fibrin layer breaks up easily, causing repeated hemorrhages, and these increase the rate of morbidity and mortality (93).

B. PATHOLOGY OF CEREBRAL VASOSPASM

Many researchers have described histopathologic changes in cerebral arteries after subarachnoid hemorrhage induced in animals and at autopsy in humans (67,68,73,94,95-114). Few have reported normal morphology of the narrowed arteries (73,115-117), and many have described a wide range of changes in the vasospastic vessels. These include swelling, proliferation, degeneration, and desquamation of the intima (95-100); the opening of endothelial junctions (95); cellular infiltration, edema, and fibrosis of the subendothelium (99,100); vacuolization, proliferation, and necrosis of smooth-muscle cells (94); infiltration of the tunica media by lymphocytes, plasma cells, and macrophages (95-96,105-106); corrugation of the internal elastic lamina (73); degeneration of perivascular nerves (104,107-111); and aggregation of platelets with the formation of thrombus intraluminally (112-113). 'Chronic structural narrowing', 'acute proliferative vasculopathy', 'constrictive endartheropathy', and 'hemorrhage arteriopathy' have been used to describe this hypothesized 'response to injury' (i.e., late cerebral vasospasm). Support for this hypothesis requires the demonstration of hyperplasia or hypertrophy of arterial smooth-muscle cells when vasospasm is angiographically visible; however, increased cross-sectional area of spastic vessels compared with control vessels in the same subject has not been found (72). Even in the case of normal vasoconstriction, thickening of the vessel wall parallels the decrease in the arterial lumen (118-119).

There is no conclusive evidence that any of these histopathologic changes causally relate to the arterial narrowing and neurologic deficits following subarachnoid hemorrhage or are the result of nonspecific reaction to vascular injury. Probably these changes in ultrastructure are neither necessary nor important in the development of cerebral vasospasm.

C. PATHOGENESIS OF CEREBRAL VASOSPASM

The pathogenesis of cerebral vasospasm after subarachnoid hemorrhage is poorly understood. The first angiographic evidence of this condition was published almost 40 years ago (7), and research into its etiology has continued ever since. It is clear that vasospasm is not an invariable concomitant of hemorrhage, as it develops only in cerebral arteries. If an assumption is made that cerebral vasospasm following subarachnoid hemorrhage is due to a single cause, it follows that this condition results from a factor produced or released in the blood entering the subarachnoid space, or from a factor in the cerebral blood vessel that is liberated or activated when the vessel ruptures, or from interaction between these factors. An agent that is involved in the development of vasospasm has to be present normally in an inactive form in cerebral circulation, and some mechanism should exist for its release after subarachnoid hemorrhage; logically, this compound should be found in the blood, the arterial wall, or the cerebrospinal fluid. Many possibilities have been investigated, and mechanical, neurogenic, myogenic, pathologic and chemical factors have been postulated in the etiology of vasospasm. Choosing among these hypotheses is not simple, there being some supporting evidence for each.

1. HYPOTHESIS OF MECHANICAL CAUSE

Almost 50 years ago Echlin produced acute, short-lived contraction of pial arteries in cats in response to mechanical stimulation (120). Some 30 years later Arutinov et al. (121) stated that only in subarachnoid hemorrhage is the arterial adventitia directly exposed to substantial amounts of blood, and postulated both mechanical and neurogenic mechanisms as causative agents of cerebral vasospasm. According to these latter researchers, blood clot acts as a mechanical factor that deforms the subarachnoid chordae attached to the vessel and at the same time irritates periadventitial nerve fibres. Later on, the same authors discredited mechanical factors as causative agents in the pathogenesis of late vasospasm (122), but there seems no doubt that mechanical factors could account for transient, acute vasospasm that is clinically insignificant in humans.

2. HYPOTHESIS OF CATECHOLAMINE AND NEUROGENIC ACTION

There is significant adrenergic perivascular innervation of the cerebral blood vessels (108, 123-125), and this is altered after subarachnoid hemorrhage (110, 111, 126-139). In cats (110, 111) and monkeys (126), catecholamine histofluorescence is markedly reduced after subarachnoid hemorrhage. As catecholamine levels increase during cerebral vasospasm, and denervation could result in supersensitivity, it seems possible that circulating catecholamines are concerned in the development of cerebral vasospasm (127). The time course of supersensitivity and the degree of innervation roughly correlate with the development of vasospasm, lending credence to this hypothesis (74, 128).

Some researchers who have induced chemical or surgical denervation found no significant difference in reactivity of the arteries to 5-hydroxytryptamine (5-HT), catecholamines, fresh or incubated blood, oxyhemoglobin, or high potassium levels (129-133). There was a suggestion that phenoxybenzamine could block clinical vasospasm (95), but the clinical response to it was disappointing (135). Other agents that affect adrenergic receptors have been tested, including phentolamine and propranolol (136) and isoproterenol or phenylephrine in combination with sodium nitroprusside (137-139). It was concluded that vasospastic cerebral arteries are unresponsive to treatment with these agents, in contrast to the response in peripheral arteries. Experimental use of surgical denervation by cervical sympathectomy to treat cerebral vasospasm (108) was neither successful nor harmful.

3. HYPOTHESIS OF MYOGENIC ACTION

The major cerebral arteries of many species, including non-human primates, dogs, cats, and rats, have no vasa vasorum; they are probably nourished by cerebrospinal fluid permeating through the minute pores in their walls (140-142), and thick subarachnoid blood clot may block the passage of sufficient nutrients and oxygen. Without glucose, the depletion of intracellular adenosine triphosphate and the consequences of this are likely to result in increased resistance to stretch and arterial rigidity. Studies in vitro showed that deprivation of glucose and oxygen for 6 hours did not make the arteries rigid, but it decreased reactivity of the vessels to 5-HT, impaired relaxation after contraction with blood, and diminished the uptake of calcium (143, 144). The authors concluded that deprivation of glucose and oxygen could decrease relaxation of contracted vessels in late cerebral vasospasm; however, studies in vivo to confirm this proposal have not been reported.

4. HYPOTHESIS OF THE ACTION OF BIOGENIC AMINES

There is some evidence that 5-hydroxytryptamine may be the agent responsible for late cerebral vasospasm (110, 111, 134, 145-148), but also some findings to the contrary (69, 149). Vasoconstriction produced by 5-HT is short-lived and tachyphylaxis develops rapidly (150). The level of 5-HT in cerebrospinal fluid decreases, reaching its minimum on day 3 after the subarachnoid hemorrhage (151). In studies of isolated baboon arteries, 501C67, a highly selective antagonist of 5-HT, only blocked arterial constriction induced by 5-HT, but not constriction induced by blood (149, 152). Therefore, it may be concluded that 5-HT is responsible for only clinically unimportant, if ever existent, early vasospasm in man.

5. PROSTAGLANDINS AND LEUKOTRIENES

It was proposed by Boullin et al. in 1979 that cerebral vasospasm is a disorder caused by an imbalance of thromboxane (TXA_2) which acts as a vasoconstrictor, and prostacyclin (PGI_2) which is a vasodilator (153). This became a popular hypothesis of cerebral vasospasm (154).

Prostacyclin (PGI_2), one of the most potent vasodilators known to man (155), is the predominant prostaglandin synthesized in healthy vascular endothelium in dogs and humans (155-162).

In the late 1970s, Asano and his co-workers devised a theory that cerebral vasospasm is initiated by clot lysis, followed by the formation of free radicals and lipid peroxidation (163-165). Free radicals and lipid hydroperoxides of 15-hydroperoxy arachidonic acid (15-HPAA) cause severe degeneration of endothelial cells and myonecrosis of the tunica media (163, 165-169). Lipid hydroperoxides also inhibit the synthesis of prostacyclin, and there is evidence of increased levels of lipid hydroperoxide and vasoconstrictive prostaglandins in the cerebrospinal fluid for up to 10 days following subarachnoid hemorrhage in humans and dogs (159-162, 164, 168, 170-178). OKY-1581 inhibits thromboxane synthetase; this agent, as well as corticosteroids and ibuprofen, causes statistically significant increases in the diameter of cerebral vessels in the single-hemorrhage canine model of cerebral vasospasm (166, 174). Both indomethacin and OKY-046 (another inhibitor of thromboxane synthetase) inhibit the contraction of canine and primate cerebral arteries to oxyhemoglobin (179, 180). Indomethacin also inhibits the contraction of tracheal smooth muscle in response to H_2O_2 in the presence and absence of endothelium (181), as well as the arterial contraction induced by xanthine oxidase (182, 183).The most potent vasoconstricting prostaglandin is thromboxane A_2, followed by $F_{2\alpha}$, E_2, and endoperoxide H_2 (165, 184-186). The clinical course of cerebral vasospasm in man parallels the concentrations of a very potent cerebral vasoconstrictor, prostaglandin $F_{2\alpha}$ (162). Prostaglandins act on receptors of smooth-muscle cells via cyclic-AMP and -GMP systems. Increase in cyclic-GMP increases the concentration of calcium within cells, by releasing it from intracellular stores or by increased membrane permeability. Relaxation is mediated by cyclic-AMP which is responsible for reduction of the intracellular concentration of calcium to less than 10^{-6} M. ONO-3708, an antagonist to the prostaglandin A_2 receptor, blocks the response of canine basilar artery to prostaglandin $F_{2\alpha}$ (187). Similarly, oxyhemoglobin inhibits the response of canine middle cerebral artery to vasodilator prostaglandin I_2 (188), and the prostacyclin analog iloprost reverses spasm induced by hemoglobin in isolated rabbit basilar artery (189).

Leukotrienes are 5-lypoxygenase products whereas prostaglandins are cyclo-oxygenase products of arachidonic acid. A few years ago it was shown that vascular tissue, including the cerebral arteries, produces leukotrienes C_4 (LTC_4), D_4 (LTD_4), and E_4 (LTE_4) (190, 191). Leukotrienes may be an important factor in cerebral vasospasm, either alone or in conjunction with prostacyclin. Leukocytes and mast cells produce leukotrienes and are found in blood clot, suggesting that leukotrienes in the blood clot may arise from inflammatory cells (192). A selective inhibitor of 5-lypoxygenase, AA-861, could retard the development of late cerebral vasospasm by decreasing the production of leukotrienes by inflammatory cells in the blood clot (193). Current investigations of the role of leukotrienes in cerebral vasospasm are yielding mixed findings (194-198).

6. BLOOD AND BLOOD PRODUCTS

In 1949 it was shown by Jackson that red blood cells contain a factor which induces the most severe chemical meningitis when injected into the cisterna magna in dogs (199). Boullin et al. (200), who studied hemorrhagic CSF from patients with ruptured aneurysm, and Okwuasaba et al. (152) who investigated CSF from patients with other CNS diseases, could only eliminate certain substances (norepinephrine, adrenaline, histamine, serotonin, acetylcholine, angiotensin II and potassium) as a cause of late cerebral vasospasm. Sasaki et al. (164) determined the nature of vasoactive substance(s) in CSF from patients with SAH, and concluded that hemoglobin, lipid hydroperoxide, and prostaglandins are concerned in late cerebral vasospasm.

Blood is removed from the subarachnoid space by lysis and phagocytosis of erythrocytes. Clot lysis starts within 24 hours, peaks during days 5-7, and declines slowly, clearing away about 75% of red blood cells in patients with SAH (168, 201). The time course of the appearance of hemoglobin from lysed erythrocytes coincides with the time course of late cerebral vasospasm in man (202). The vasoactive properties of incubated blood increase within 36 hours, reach their maximum on day 3 and continue for up to 14 days. The contractile activity of the lysed blood is dose-dependent on the concentration of a substance that has a molecular weight and an ultraviolet spectrum similar to those of hemoglobin (163, 165, 203, 204). Intact erythrocytes are not vasoactive, whereas lysed red cells alter the tension of isolated dog basilar artery (203). Hemoglobin causes contraction of smooth muscle in vitro (205-208), but purified hemoglobin is only one-third to half as vasoactive as hemolysed blood (204, 209).

When the lysis occurs, oxyhemoglobin (oxyHb) is released in the subarachnoid space; there it undergoes spontaneous oxygenation to methemoglobin (metHb), a reaction that produces superoxide anion (210-212). Superoxide is converted by superoxide dismutase (SOD) to hydrogen peroxide (H_2O_2), which under normal conditions is inactivated by catalase or glutathione peroxidase. However, when iron complexes (Fe^{++}, Fe^{+++}) are present, superoxide and hydrogen peroxide produce very active singlet oxygen and hydroxyl radicals by the so-called Haber - Weiss reaction. Superoxide may also react with hydroperoxides of polyunsaturated fatty acids, producing alkoxyl radical. These active free radicals - hydroxyl radical, singlet oxygen, and alkoxyl radical - initiate peroxidation of polyunsaturated fatty acids, producing fatty-acid free radicals, which combine with oxygen to become lipid peroxide. Lipid peroxide produces more free radicals, starting a chain reaction of lipid peroxidation (212-214). Lipid peroxides and/or free radicals generated by non-enzymatic reaction within the clot stimulate C-12 lipoxygenase within the clot, and this in turn stimulates C-5 lipoxygenase in leukocytes and the arterial wall. Leukotrienes are 5-lipoxygenase products, and prostaglandins such as PGH_2, thromboxane A_2, and $PGF_{2\alpha}$ are the cyclo-oxygenase products of arachidonic-acid metabolism. Products of C-12 and C-15 lipoxygenase give rise to other vasoactive products also. Lipid hydroperoxides also inhibit synthesis of the most potent vasodilator, prostacyclin.

There is considerable evidence that lipid peroxidation is probably involved in late cerebral vasospasm (162). However, establishment of a link between the non-enzymatic and enzymatic lipid peroxidation pathway would be the most important piece in the jigsaw to solve the puzzle of the pathogenesis of cerebral vasospasm (215).

D. CLINICAL MANAGEMENT OF CEREBRAL VASOSPASM

Although some progress has been made, cerebral vasospasm following the rupture of an intracranial aneurysm is still one of the biggest problems in neurosurgical therapy. Wilkins has regularly reviewed methods of prevention and treatment of cerebral vasospasm (216, 217), and in his most recent review, in 1986, came to the pessimistic conclusion that the problem is still unsolved.

The simplest way to prevent or treat cerebral vasospasm would be the removal of the agent(s) responsible for its development (218-223). Removal of perivascular clot in the subarachnoid space by early operation, and cisternal drainage and irrigation, reduces mortality and morbidity of cerebral vasospasm (40) and diminishes the risk of rebleeding (224). Some neurosurgeons have questioned the usefulness of early operation, because of the fear that the brain lesion caused by arterial rupture could be worsened by surgical manipulation of the diseased vessel (225). Ljunggren et al. concluded that an early removal of blood clot does not eliminate the risk of cerebral ischemia from cerebral vasospasm (226). However, late operation also carries a risk: further rebleeding may occur, resulting in higher rates of morbidity and mortality (227). The above arguments make the decision for an early or late operation much more difficult.

Some possible modes of therapy against cerebral vasospasm have been summarized by Towart (206) and Sano (214). A few of them are:

1. Hemodynamic improvement of impaired cerebral circulation, by increasing the systemic arterial pressure with dopamine or by inducing hypervolemia (65).

2. Destruction of free radicals, with scavengers such as vitamin E or mannitol.

3. Inhibition of thromboxane synthesis by administering OKU-1581 and OKU-

046; and/or stimulation of prostacyclin synthesis with prostacyclin and its analog iloprost.

4. Suppression of platelet aggregation, and pharmacologic modification of prostaglandin synthesis with steroids to suppress phospholipase A_2.

5. Stimulation of adenyl cyclase with isoproterenol or salbutamol.

6. Inhibition of phosphodiesterase with papaverine, aminophylline, chlorpromazine, hydralazine and reserpine.

7. Reduction of calcium-induced vasospasm with antagonists such as nimodipine, nifedipine, nicardipine and diltiazem.

The list goes on and on, but the most promising therapy uses antagonists of calcium influx in the prevention and treatment of late cerebral vasospasm, for example the dihydropyridines. These agents are most beneficial in cases of subarachnoid hemorrhage in which late cerebral vasospasm is likely to develop. However, the benefit is probably not due to preventing the influx of calcium into cerebrovascular smooth-muscle cells, but rather the protection of marginally anoxic brain tissue. Some other vasorelaxant agents may be of benefit by blocking a pathway for the development of tension in smooth-muscle cells; for example, prostacyclin analogs or agents that block the effects of prostaglandin $F_{2\alpha}$ probably antagonize the effects of oxyhemoglobin in this way. Some other agents from this category include glycerol trinitrate, which relaxes spastic primate arteries with or without endothelium (228), and calcitonin gene-related peptide, which has been shown angiographically to reverse vasospasm induced by subarachnoid hemorrhage in dogs (229).

CEREBRAL VASOSPASM

PART II

A. RATIONALE FOR PRESENT APPROACH

It has been hypothesized that delayed cerebral vasospasm results from a sustained contraction of the cerebrovascular smooth-muscle cells. It is important to understand the signal transaction pathways in the plasma membranes of the cerebral arterial smooth-muscle cells under physiologic and/or pathologic conditions if one is to determine the mechanisms that control vasoconstriction and vasodilation. Membrane ion channels are a primary factor in this signal transaction (230). It appears that calcium influx is important in initiating and maintaining contraction of cerebrovascular smooth muscles (231, 232). Calcium antagonists, such as dihydropyridines (233), are used in the clinical management of cerebral vasospasm. Their mode of action is probably not the prevention of calcium influx into cerebrovascular smooth-muscle cells, but rather the protection of hypoxic brain tissue. Study of the calcium-activated potassium channels is very important for understanding the signal transaction mechanism of contraction of cerebral arterial smooth-muscle cells.

Direct studies of the ion channels in the plasma membrane of cerebrovascular smooth muscle are technically difficult, because of the properties of smooth-muscle cells: they are contractile, electrotonically coupled, among the smallest cells of the body, and are surrounded by a thick layer of the connective tissue of tunica adventitia (230).

Until now, only the single microelectrode clamp technique has been used to describe voltage-clamp data from cerebrovascular smooth muscle. The question to be addressed is: How well is the membrane potential controlled in an elongated cell that is electrically coupled to its neighbors? The membrane potential at the recording site might not represent the actual membrane potential of the whole cell, because of the cell length and its electrical coupling; this error in the voltage control could be quite high and very difficult to assess and correct (234). To reduce this problem of voltage inhomogeneity, the best approach is to use the patch clamp recording technique on single isolated cerebrovascular smooth-muscle cells. The elimination of electrical coupling of the cell under investigation to its neighbors dramatically increases voltage homogeneity, but the problem might still exist because of the shape of these cells. For example, isolated smooth-muscle cells of rat basilar artery are not larger than 150 μm long, 5 μm wide, and 2 μm thick. This is a very small cell, and even if the patch-clamp electrode is placed at one end, control of the opposite end will be within 90%. As most seals are formed at the plumpest part (the middle) of a cell, a patch electrode could control the end points more than 95% (235). This degree of voltage control is adequate for interpreting currents.

An enzymatic dissociation of cerebral arteries is used to isolate single smooth-muscle cells that are later used in patch-clamp experiments. There are some advantages and disadvantages in working with single, isolated cells. Advantages include the ability to study whole-cell and single channel currents on one cell type, using current- and voltage-clamp techniques, as well as to alter the intracellular solution. Disadvantages include the potential hazard of loss of the cell's characteristics, due to cellular dissociation or during successive passages in culture. Smooth-muscle cells are very prone to both of these disadvantages. Therefore, the cells have to be characterized by methods such as application of a stain positive for α-actin, which is a specific marker of smooth-muscle cells, and use of only the primary culture.

B. PATCH CLAMPING: WHOLE-CELL AND SINGLE-CHANNEL CURRENTS

Ion channels are macromolecular pores that evolved, together with a cell lipid biomembrane, over three-billion years ago. Today, ionic channels are regarded as excitable elements in the membranes of excitable cells (236).

The extracellular patch-clamp method was used for the first time 15 years ago, to detect the currents in single ionic channels (237). In patch-clamp technique a small, clean, heat-polished, solute-filled glass pipette with a tip opening of 1-2 μm in diameter is pressed against the cell membrane, forming a high-resistance seal between the membrane and the glass when suction is applied to the pipette's interior (238). This mode of recording is known as the cell-attached membrane patch; and the tight pipette/membrane seal, with resistances of 10-100 GΩ, is known as a giga-seal. After giga-seal formation additional suction applied to the pipette ruptures the cell membrane but maintains attachment of the pipette to the cell. A low-resistance pathway is formed between the interior of the pipette and the interior of the attached cell.

Patch clamping has some advantages over conventional microelectrodes: the patch electrodes are less damaging to the cell, and the pipette solution exchanges with the intracellular solution, thereby controlling the composition of the intracellular solution. Calcium currents and delayed-rectifier potassium current run down during the whole-cell recording, an effect that is postulated to result from the loss of some soluble cytoplasmic factor(s) during cell dialysis against the pipette contents. The configuration of whole-cell recording allows control and accurate recording of the membrane potential across the surface membrane as well as voltage clamping of a small cell, such as the cerebrovascular smooth-muscle cell. This is in contrast to the inhomogeneous voltage of electrically coupled cells in intact vessels.

The patch-clamp technique also permits the recording of single-channel current from isolated membrane patches. After the mechanically stable giga-seal has been established, the pipette is pulled away from the cell membrane; this forms a membrane vesicle across the pipette tip. A very short crossing of the air/water interface by the pipette tip destroys remnants of the cell except the patch across the tip and produces an inside-out patch, in which the cytoplasmic cell surface is exposed to the bathing medium and the extracellular surface is in contact with the pipette solution. An outside-out patch is formed when the pipette is pulled away from a cell that is already in the whole-cell recording mode: the extracellular surface is exposed to the bathing medium, and the intracellular surface is facing the pipette solution. These cell-free giga-sealed membrane patches that close the opening of the pipette tip allow one to record single-channel current in controlled media and to change the solution during the recording.

The cell-attached single-channel recording mode is most useful for studying ion channels that appear to depend on an intracellular factor at present unknown. An inside-out patch is used for applying channel blockers to the cytoplasmic side of the membrane which is facing the bathing solution. When the solution is exchanged, the effects of a channel blocker are washed away if the block is reversible.

C. PREVIOUS STUDIES OF ELECTROPHYSIOLOGY

Many questions concerning the mechanism of contraction of vascular smooth muscle remain unanswered. By regulating the amount of intracellular free calcium, the smooth-muscle cells regulate the amount of contraction of the vessel and consequently determine the diameter of its lumen. The exact nature of membrane-based signal transduction during the increase in intracellular free calcium is not known, but the two likely mechanisms are: (a) an influx of calcium into the cell via voltage-dependent channels, receptor-operated channels, and/or stretch-dependent channels, and (b) release of sequestered calcium from intracellular stores (239, 240). An influx of extracellular calcium into the cell is essential for initiating cerebrovascular smooth-muscle contraction (231). A postulated mechanism that couples electrical, chemical, and mechanical signals to cerebrovascular arterial smooth-muscle contraction via voltage-dependent, receptor-operated, and mechanically sensitive membrane-ion channels has not been delineared.

The properties of smooth-muscle cells in the cerebral and other arteries differ (241). Consequently, only data from cerebrovascular smooth muscle can be used to investigate the pathogenesis of late cerebral vasospasm.

VOLTAGE-DEPENDENT CHANNELS

Very little research has been done on the electrophysiology of cerebrovascular smooth muscle. Using technically very difficult, single-microelectrode clamp technique, Hirst et al. (242) detected five voltage-dependent currents in proximal segments of rat middle cerebral arteries: two calcium currents, two outward currents that were activated at depolarizing potentials, and an inward rectifying current that showed an increase in membrane conductance for the membrane potentials more negative than -80 mV. The density of inward currents was greater in proximal than in distal segments of the arterioles (243). Nosko et al. provided additional evidence that smooth-muscle cells from monkey and human middle cerebral arteries have voltage-dependent calcium channels; calcium-free medium or calcium channel blockers relaxed arteries that had contracted in response to a high external concentration of potassium (244, 245).

Voltage-dependent calcium channels have been identified in the plasma membranes of many cell types (246) and some vascular smooth-muscle cells (247, 248), with the patch-clamp technique, but studies of cerebrovascular smooth- muscle cells with this technique have not been reported. Thus it was necessary to determine whether different types of calcium channels have different sensitivities to clinically used calcium-channel blockers, such as dihydropyridines.

RECEPTOR-OPERATED CHANNELS

Noradrenalin is a neurotransmitter of the sympathetic autonomic nervous system that causes contraction in endothelium-free rat aorta (249). It is known that noradrenaline alters membrane conductances by binding to a receptor that (a) opens a receptor-operated calcium channel (250) or (b) indirectly influences membrane conductances via second messengers (249), but the final details of its mechanism remain to be worked out. Other substances, including some neurotransmitters (e.g. dopamine), cause membrane depolarization and contraction in rabbit basilar artery, effects that can be stopped by agents that block the calcium channels (251).

MECHANICALLY SENSITIVE CHANNELS

Vascular smooth-muscle contracts and relaxes in response to increases and decreases in stretch and tension. Temperature-sensitive, stretch-activated membrane ion channels may be a pathway of entry for calcium into the cell. Rousseaux et al. described correlation between hyperthermia, SAH, and severe symptomatic cerebral vasospasm in up to 88% of their patients (252). It is possible that hyperthermia associated with SAH enhances smooth-muscle tone through stretch-activated membrane ion channels. Using the patch-clamp technique, a mechanically sensitive channel was discovered in primary cultures of endothelial cells from pig aorta (253) and in freshly dissociated smooth-muscle cells of toad stomach (254).

D. OBJECTIVES

It has been proposed that delayed cerebral vasospasm following subarachnoid hemorrhage results from a sustained contraction of the smooth-muscle cells residing in the vessel wall. The objectives of this project were:

1. To establish the feasibility of isolating single vascular smooth-muscle cells from rat cerebral arteries.

2. To verify that isolated smooth-muscle cells retain morphologic and physiologic properties of cells in the intact tissue.

3. To show that the patch-clamp technique can be applied to the study of ion channels in these cells.

4. To characterize membrane properties of isolated smooth-muscle cells using whole-cell voltage patch-clamp technique.

5. To examine the mechanism of action of vasoactive substances (i.e., oxyhemoglobin and bilirubin) which may be responsible for the development of late cerebral vasospasm in man.

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CHAPTER II.
FREE RADICALS MEDIATE ACTIONS OF OXYHEMOGLOBIN ON CEREBROVASCULAR SMOOTH MUSCLE CELLS

INTRODUCTION

On the basis of incidental autopsy findings, it has been estimated that five million North Americans harbor aneurysms of their cerebral circulation. Rupture of aneurysms produces about 30,000 cases of subarachnoid hemorrhage in North America each year (1). Among the patients surviving the initial hemorrhage with neurological impairment, almost all will develop significant constriction of cerebral arteries (vasospasm) more than five days after bleeding (2). Consequences of cerebral vasospasm include delayed ischemic deficits and death. In survivors, spontaneous reversal of vasospasm occurs within 7-21 days. The pathogenesis of cerebral vasospasm is poorly understood and there is currently no effective therapy for its prevention or reversal (3).

Several observations suggest that hemoglobin plays a role in producing vasospasm. The time course of the appearance of hemoglobin coincides with the time course of vasospasm in patients (4). Hemoglobin has been shown to cause contraction of vascular smooth muscle in vitro (5-8). In additional support of the hypothesis that hemoglobin is the spasmogen, the substance in incubated erythrocytes which causes contraction of smooth muscle has a molecular weight and ultraviolet spectrum similar to hemoglobin (9).

Elucidation of the mechanism of action of oxyhemoglobin on cerebrovascular

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A version of this chapter has been published. Steele, Stockbridge, Maljkovic & Weir 1991. *Circulation Research* 68:416-423.

smooth muscle may lead to a treatment for vasospasm. A possible mechanism for the action of hemoglobin arises from the observation that oxyhemoglobin (Fe^{2+} state), in its auto-oxidation to methemoglobin (Fe^{3+} state), produces superoxide anions (O_2^-) (10-12), and, secondarily, other free radicals. Consistent with this hypothesis, oxyhemoglobin is the form of hemoglobin which has been shown to cause contraction of smooth muscle (5, 8). Free radicals can participate in such reactions as the peroxidation of polyunsaturated fatty acids. Indeed, peroxidation rates in incubated blood rise over 3-5 days and remain high for at least 14 days (12).

The mechanism for free radical production by oxyhemoglobin is believed to be: (10, 11)

$$\text{Hb-Fe}^{2+} + O_2 \rightarrow \text{Hb-Fe}^{3+} + O_2^-$$

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + \dot{O}_2$$

$$H_2O_2 + \text{Hb-Fe}^{2+} \rightarrow OH^{\cdot} + OH^- + \text{Hb-Fe}^{3+}.$$

Studies of the electrophysiological properties of vascular smooth muscle cells in intact arteries are difficult to interpret because of the electrical coupling between the smooth muscle cells (13, 14) and because of the interactions between the endothelial cells and the smooth muscle cells. In the present study, such complications were avoided by the use of isolated single cerebrovascular smooth muscle cells. Single cells were isolated from the basilar artery of the rat by enzymatic dispersion. Primary isolates were chosen in lieu of cells grown in tissue culture since cultured smooth muscle cells may dedifferentiate (15). The membrane properties of the cells were studied using the whole-cell patch clamp technique (16) and cell viability was measured by fluorescein diacetate uptake (17). The effects of oxyhemoglobin on the cells were studied and the ability of various agents to either block the actions of oxyhemoglobin or to mimic it were investigated.

MATERIALS AND METHODS

Cell preparations. Female Sprague-Dawley rats two months old were anesthetized with halothane and decapitated. The basilar arteries were removed to a solution consisting of (mM) NaCl 130, KCl 5, $CaCl_2$ 0.8, $MgCl_2$ 1.3, glucose 5, HEPES 10, penicillin (100 U/ml) and streptomycin (100 mg/l). The arteries were cleaned of connective tissue and small side branches. The arteries were moved to a solution in the which the $CaCl_2$ concentration was reduced to 200 μM and to which was added collagenase (Type II, 500 mg/l), elastase (500 mg/l), hyaluronidase (Type IV-S, 500 mg/l) and deoxyribonuclease I (100 mg/l). The arteries were cut into approximately 200 μm rings and incubated for 1 h at room temperature. The rings were transferred to fresh solution (200 μM $CaCl_2$) containing trypsin inhibitor (500 mg/l) and deoxyribonuclease I (100 mg/l) and then triturated gently using a Pasteur pipette. Cells were plated on pieces of glass cover slips and stored at 4°C in saline containing essentially fatty acid free bovine serum albumin (2 g/l) and $CaCl_2$ (800 μM). The isolated cells stained positive for α-actin, a specific marker for smooth muscle cells (18). Elongated cells contracted upon exposure to 40 mM potassium saline and upon exposure to a variety of agonists: serotonin (500 nM); angiotensin II (10 μM); prostaglandin $F_{2\alpha}$ (10 μM) and prostaglandin E_1 (600 μM). Under voltage-clamp, the cells had an input resistance of 5-10 GΩ and a capacitance of 20-30 pF and exhibited inward and outward voltage-dependent currents. These observations showed that the cells were undamaged by the isolation procedure.

Undifferentiated murine neuroblastoma cells, clone N1E-115, were grown in Dulbecco's modified Minimum Eagle Medium supplemented with 10% fetal calf serum and gentamycin (60 mg/l) in a humidified atmosphere with 5% CO_2 at 37°C. All tissue culture supplies were purchased from GIBCO (Calgary, AB). Cells were used 48-72 h post-plating.

Techniques. Whole-cell membrane currents were recorded by the patch electrode voltage clamp technique (16) using an Axopatch 1B or 1C patch clamp amplifier (Axon Instruments, Burlingame, CA). The patch pipettes had resistances of 1-4 MΩ. The input resistance was determined from small, hyperpolarizing voltage steps. Series resistance compensation was not employed. Since the leakage conductance increased dramatically following certain treatments, the series resistance error frequently exceeded 5 mV. This error would cause an underestimation of the leakage and outward currents reported here. Data acquisition, storage and analysis were performed with a dedicated laboratory computer.

The viability of the cells was assessed using fluorescein diacetate. In viable cells, membrane-permeant, non-fluorescing fluorescein diacetate is enzymatically cleaved to form impermeant, but highly fluorescent fluorescein (17). These results represent a lower limit on the percentage of cells sustaining damage as it frequently was noted that some cells which had membrane blebs were fluorescent.

Solutions. For electrical recording, the normal bath solution was (mM) NaCl 130, KCl 5.4, $MgCl_2$ 1.2, $CaCl_2$ 1.8, HEPES 10, glucose 5.2 and the pH was adjusted to 7.4 with NaOH. The normal pipette solution was (mM) KCl 139, $MgCl_2$ 0.5, EGTA 2, HEPES 10, glucose 10 and the pH was adjusted to 7.4 with KOH. Experiments with Fenton's reagent (500 µM H_2O_2 + 1 mM Fe^{2+}; 18) were done in a bath solution containing Tris buffer adjusted to pH 6.0 to favor the solubility of Fe^{2+} over Fe^{3+}. Although buffers are hydroxyl radical scavengers (19), control experiments in unbuffered solutions showed that neither Tris nor HEPES exerted protective effects against oxyhemoglobin.

Rat hemoglobin was prepared as a 1 mM solution in 150 mM NaCl. Oxyhemoglobin was produced by reduction of hemoglobin by 10 mM $Na_2S_2O_4$ (20). Methemoglobin was produced by oxidation of hemoglobin by 2 mM $K_3Fe(CN)_6$. Either reagent was removed by dialysis against 1000 volumes of 150 mM NaCl with several solution changes over 36 h at 4°C, Aliquots of the hemoglobin solutions were stored frozen for no more than two weeks. For spectrophotometric measurements of concentration (21), hemoglobin solutions were diluted 100-fold in phosphate buffer (78 mM Na_2HPO_4 + 22 mM KH_2PO_4) at pH 7.3.

Xanthine (1 mM) + xanthine oxidase (10 U/l) was used as a source of superoxide anions. Fenton's reagent or $CuCl_2$ (100 μM) + ascorbic acid (100 μM, to reduce Cu^{2+} to Cu^+) + H_2O_2 (100 μM) were used as sources of hydroxyl free radicals (22). Since Fe^{2+} was found to block potassium currents, the Cu^+-based system was used for electrophysiological experiments.

Superoxide dismutase from bovine erythrocytes was dialyzed to remove the potassium salts. Inactivation of superoxide dismutase was achieved by boiling for one minute (23). Catalase from bovine liver was used. All agents were purchased from Sigma (St. Louis, MO).

Statistical analysis. Where appropriate, values are reported as means ± s.e.mean. A Student's t-test was used to compare control and experimental values of input resistance (Table 1). For viability experiments, a Yates-corrected χ^2 test was used to compare control conditions with oxyhemoglobin exposure or oxyhemoglobin exposure to oxyhemoglobin + free radical scavengers. In all cases, a $P < 0.05$ was considered significant.

RESULTS

Oxyhemoglobin has multiple effects on cerebrovascular smooth muscle cells. Relaxed, phase-bright cerebrovascular smooth muscle cells were studied. Within several minutes of exposure to oxyhemoglobin (5 μM), the cells shortened (Fig. 1, middle row). None of the cells elongated again following washout of oxyhemoglobin; however, in our experience, these cells never lengthened out following contraction to any agonist, probably because the normal restoring force is missing in cells. Upon continued exposure to oxyhemoglobin, the cells developed membrane blebs (Fig. 1, lower row). If the exposure was no more than a couple of minutes, approximately 50% of the cells reabsorbed the blebs and continued to appear phase bright. In contrast, methemoglobin (100 μM) was without visible effects. Control experiments with the agent used to reduce methemoglobin to oxyhemoglobin, $Na_2S_2O_4$ (1 mM), showed no effects.

Under whole-cell voltage clamp, exposure to oxyhemoglobin (5 μM), caused 68% of the smooth muscle cells to exhibit changes in outward currents and membrane resistance (Fig. 1, Table 1). Within several minutes, the magnitude of the outward currents began to increase. The currents continued to increase over the next several minutes and attained a final magnitude up to 10-fold over control (Fig. 1). The outward currents were carried by potassium ions since they were absent when the potassium in the pipette solution was replaced with the impermeant ion, cesium (24). Similar increases in the potassium currents were observed when using pipettes with higher free calcium and also occurred in response to 1 mM caffeine.

The increase in the potassium currents was followed by a sudden, dramatic decrease in the membrane resistance. The resistance decreased approximately 20-fold, demonstrating an extremely large increase in the permeability of the surface membrane (Fig. 1; Table 1). The remaining current showed no voltage- or time-dependence and the zero-current potential, which gives a measure of the reversal potential, was approximately 0 mV. The effect of oxyhemoglobin was dose-dependent with 100 μM causing approximately half of the cells to lose the ability to concentrate fluorescein in less than 30 minutes (Table 2).

Oxyhemoglobin (10 μM) was without effect on cultured mouse neuroblastoma cells (Fig. 2). None of the cells developed membrane blebs and there was no effect on the potassium currents or the membrane resistance (control: 1.2 ± 0.2 GΩ; oxyhemoglobin: 1.0 ± 0.2 GΩ; n = 9). There was a small transient depression of the inward currents in the continued presence of oxyhemoglobin.

Free radicals mediate the effects of oxyhemoglobin. To investigate the mechanism whereby oxyhemoglobin affected the cells, the actions of other agents were studied. Methemoglobin (100 μM) had no effect (Tables 1 and 2). Control experiments with $Na_2S_2O_4$ (1 mM) also showed no effects.

Superoxide dismutase (170 U/ml) was found not to protect against the electrophysiologically observed effects of oxyhemoglobin (Table 1). In viability tests (Table 2), superoxide dismutase produced dose-dependent increases in cell survival at concentrations up to 100 U/ml. At 100-1000 U/ml, superoxide dismutase in the presence of oxyhemoglobin produced survival rates which plateaued well below control levels in the absence of oxyhemoglobin. In contrast, catalase (300 U/ml) protected against the effects of 100 μM oxyhemoglobin in both electrophysiological experiments (Table 1) and viability tests (Table 2). The best results, a complete block of the deleterious effects of oxyhemoglobin, were obtained with both catalase (300 U/ml) and superoxide dismutase (170 U/ml).

Further experiments to investigate the actions of hydrogen peroxide, superoxide anions and hydroxyl radicals were done. Application of either hydrogen peroxide (500 μM) or xanthine (1 mM) + xanthine oxidase (10 U/l) increased the potassium currents but did not affect the membrane resistance (Table 1) or cell viability (Table 2). Exposure of the cells to $CuCl_2$ (100 μM) + ascorbic acid (100 μM) + H_2O_2 (100 μM) caused the potassium currents to increase followed by a dramatic decrease in the input resistance (Table 1). In viability experiments, Fenton's reagent proved to be very cytotoxic (Table 2). Viability tests with dimethyl sulfoxide (0.5%), a potent hydroxyl radical scavenging agent (25), showed it to be substantially effective in blocking damage produced by oxyhemoglobin or by Fenton's reagent (Table 2). It was not possible to use dimethyl sulfoxide in patch clamp experiments as stable seals could not be maintained.

DISCUSSION

Oxyhemoglobin was found to have multiple effects on isolated single cerebrovascular smooth muscle cells of the rat. The cells were observed to contract within several minutes of exposure to oxyhemoglobin. In agreement with an earlier report (8), this observation showed that oxyhemoglobin exerted a direct action on the smooth muscle cells which was independent of endothelial cells. As well, this observation suggests that oxyhemoglobin may trigger an increase in intracellular free calcium since a rise in intracellular free calcium is necessary to initiate contraction in vascular smooth muscle (14). Following contraction, the cells developed membrane blebs indicating severe damage to the cell membrane.

Under whole-cell voltage clamp, two effects of oxyhemoglobin were recorded. The first response was a very large increase in the outward currents. The increase in the magnitude of the currents can be attributed to an increase in the number of calcium-activated potassium channels which were open. Vascular smooth muscle cells have been reported to have a high density of calcium-activated potassium channels (26). In the present study, the existence of calcium-activated potassium channels was shown with high calcium pipette solutions and by the fact that caffeine caused an increase in the magnitude of the currents. Caffeine increases the concentration of intracellular calcium by releasing it from intracellular stores (27). As the intracellular concentration of free calcium increases, calcium-activated potassium channels remain open a larger fraction of the time (28) and consequently, the magnitude of the macroscopic currents increases. Thus, these ion channels can serve as an indicator of the intracellular free calcium concentration. The increase in the currents seen with oxyhemoglobin can thus be explained by an increase in the intracellular concentration of calcium. Such an increase in intracellular free calcium could have brought about the other effects seen with exposure of the cells to oxyhemoglobin, *viz.* contraction, membrane blebs, increase in the outward currents, increase in membrane conductance and cell death.

The mechanism by which oxyhemoglobin produced a rise in intracellular calcium is unclear. The observation that oxyhemoglobin did not have the same effect on neuroblastoma cells suggests that the mechanism may involve intracellular calcium stores present in smooth muscle cells but absent in neuroblastoma cells.

The second response observed under voltage clamp was a decrease in the input resistance of the cells upon exposure to oxyhemoglobin. This indicated a very large increase in the conductance of the surface membrane. This remaining leakage current had a zero-current potential of 0 mV indicating that the remaining conductance was nonselective for ions. A previous report of intracellular recordings obtained from smooth muscle cells in intact arteries has shown that oxyhemoglobin produced an increase in the input resistance (29). This is the opposite to what was observed in the present study. A possible explanation for this discrepancy is that the intracellular concentration of calcium increased following exposure of the intact arteries to oxyhemoglobin, as is suggested by the present results. This would lead to a decrease in cell-to-cell coupling (30) and consequently a higher input resistance. The loss of the smooth muscle cells' ability to concentrate fluorescein after exposure to oxyhemoglobin was another indication of the great rise in membrane permeability.

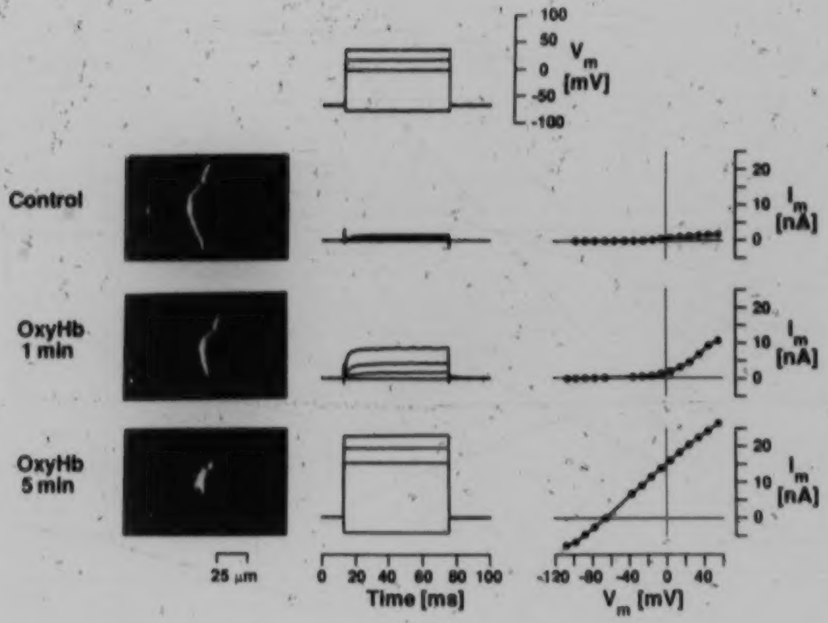
There is speculation in an earlier report that the generation of free radicals by oxyhemoglobin is responsible for its effects on smooth muscle (5). The data presented in this report support this hypothesis. It was found that methemoglobin, which does not generate free radicals, had no effect. In agreement with this result, others have reported that oxyhemoglobin is more effective than methemoglobin in producing arterial contraction (5, 6). Viability experiments with superoxide dismutase support the sequence of reactions shown in the Introduction. Superoxide dismutase was partially effective in improving viability of cells exposed to oxyhemoglobin. This improvement would have been expected if superoxide anions produced deleterious effects directly or by leading to the formation of hydroxyl radicals. At concentrations of greater than 100 U/ml, superoxide dismutase failed to produce further improvements in viability. This may have been because hydrogen peroxide produced by superoxide dismutase participated in the oxyhemoglobin-mediated formation of hydroxyl radicals. Hydrogen peroxide alone was without effect, as were superoxide radicals produced by xanthine + xanthine oxidase (with no Fe^{2+} to produce hydroxyl radicals secondarily). Interestingly, both of these treatments caused an increase in the outward potassium currents. Since superoxide anion spontaneously dismutates to hydrogen peroxide, it may be that hydrogen peroxide was responsible for the increase in the potassium currents observed with xanthine + xanthine oxidase. Also, catalase and dimethyl sulfoxide, which both eliminate hydroxyl radicals, were found to protect against oxyhemoglobin. Further support for the involvement of hydroxyl radicals came from experiments in which hydroxyl radicals were generated by the combination of metal ions and hydrogen peroxide. Both hydroxyl radicals and oxyhemoglobin caused similar effects, *viz.* an initial increase in the outward currents followed by a very large inrease in the membrane conductance and cell death. Taken together, these results suggest that free radicals, in particular hydroxyl radicals, were responsible for the effects of oxyhemoglobin on the smooth muscle cells.

Superoxide anions have been reported to accelerate the degradation of endothelium-derived relaxing factor (EDRF) (31). However, in contrast to experiments with arterial rings, in which hemoglobin had access to intra- and extralumenal surfaces (32), when oxyhemoglobin was applied extralumenally to intact arteries, endothelium-mediated responses to acetylcholine (33) and KCl (34) were unattenuated and yet a hemoglobin-induced contraction persisted which was endothelium-independent (8, 34). Thus the effect of hemoglobin on EDRF removal may not be very important in producing vasospasm observed with subarachnoid hemorrhage.

Spasm of cerebral arteries can be a devastating late complication of hemorrhagic stroke. Some evidence has supported the hypothesis that vasospasm results from oxyhemoglobin released from red blood cells in the clot (4, 5-9, 35). The results reported here, using single isolated smooth muscle cells obtained from cerebral arteries of the rat, support this hypothesis and the suggestion that production of free radicals by oxyhemoglobin is involved in the pathogenesis of vasospasm via direct effects on smooth muscle cells. In the present study, it was found that oxyhemoglobin caused (1) contraction, (2) membrane blebs, (3) a very large increase in the outward potassium currents, (4) an extremely large decrease in the membrane resistance and

(5) cell death. All of these effects could result from a large increase in the concentration of intracellular free calcium. Experiments with methemoglobin, superoxide dismutase, catalase, and dimethyl sulfoxide suggested that the effects were the result of the generation of free radicals. Further support for this conclusion was obtained from experiments showing that hydroxyl radicals generated by metal ions plus hydrogen peroxide caused identical effects to those observed with oxyhemoglobin. Together with the evidence that superoxide anions accelerate the degradation of EDRF (31), the present study suggests that a therapeutic strategy aimed at the reduction in free radicals could be effective in alleviating chronic cerebral vasospasm resulting from subarachnoid hemorrhage.

Figure II-1. Actions of oxyhemoglobin on an isolated cerebrovascular smooth muscle cell. **Column 1:** A single cell was photographed under an inverted microscope with Nomarski optics. The control cell length was ~100 μm. On exposure to 5 μM oxyhemoglobin, the cell contracted within about 1 min and showed further contraction and membrane blebs within 5 min. **Column 2:** Membrane currents were recorded from a different single cell using the patch electrode voltage clamp technique. Voltage clamp command waveforms are shown in the top set of superimposed traces. Shown below are three sets of membrane currents which were not corrected for leakage currents. Four current traces are superimposed in each set. One minute after application of oxyhemoglobin to the solution bathing the cell the magnitude of the outward potassium currents (upward deflections) increased dramatically. The most likely explanation is that the intracellular calcium concentration increased and thus increased the number of calcium-dependent potassium channels that were open. After several more minutes the electrical resistance of the membrane decreased dramatically. **Column 3:** Current-voltage plot of the data in column 2. Brief exposure to oxyhemoglobin caused an increase in the magnitude of the potassium currents over control values. Prolonged exposure caused an extremely large increase in the leakage conductance of the membrane as evidenced by the increase in the slope of the line at 5 min. For this particular cell, the input resistance (an indicator of membrane permeability) in the control was 3.0 GΩ, after 1 min of oxyhemoglobin it was 0.1 GΩ and after 5 min it was 0.006 GΩ. This indicates that the permeability of membrane increased dramatically following application of oxyhemoglobin.


100
50
0
-50
-100
V_m [mV]
Control
OxyHb 1 min
OxyHb 5 min
20
10
0
I_m [nA]
25 µm
0 20 40 60 80 100
Time [ms]
-120 -80 -40 0 40
V_m [mV]

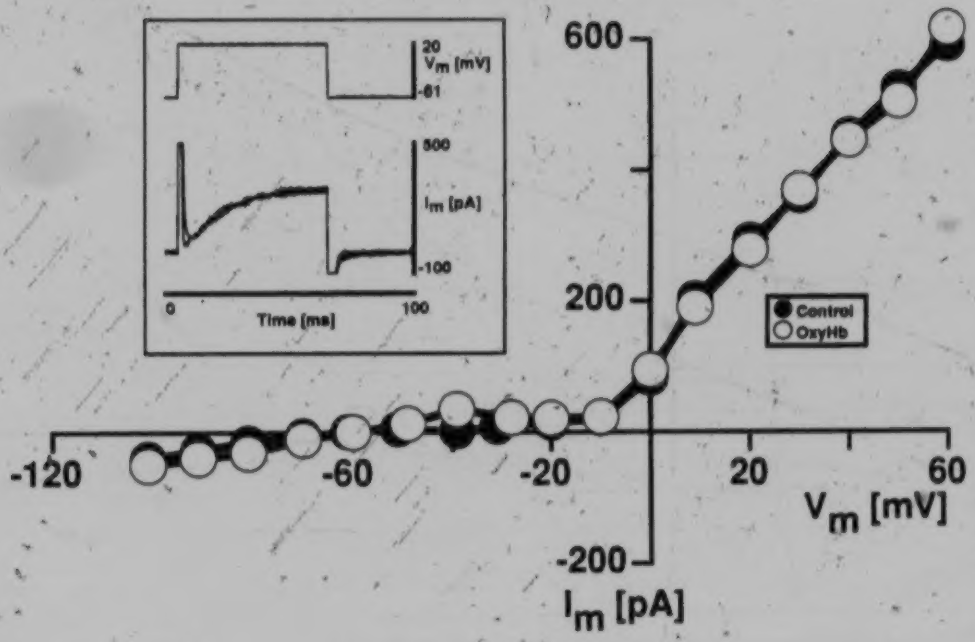


Figure II-2. Current-voltage plot and current records (inset) for an N1E-115 mouse neuroblastoma cell exposed to oxyhemoglobin (10 μM) for 5 min. Current records were not corrected for leakage currents.

Agent *(n)*	R_{in} (GΩ ± S.E.M.)		Effect on I_K
	Control	Expt	
OxyHb *(21/31)*	4.0 ± 2.4	0.2 ± 0.7*	eliminated
MetHb *(7)*	4.5 ± 1.5	4.0 ± 3.3	unchanged
OxyHb + catalase *(6)*	6.0 ± 3.4	3.8 ± 3.9	unchanged
OxyHb + SOD *(5/7)*	3.8 ± 1.5	0.2 ± 0.3*	eliminated
X + XO *(5)*	8.7 ± 5.6	2.3 ± 2.8	increased
H_2O_2 *(4)*	4.6 ± 2.8	4.4 ± 3.5	increased
$H_2O_2 + Cu^{++}$ *(4)*	5.8 ± 3.0	0.3 ± 0.2*	eliminated

Table II-1. Summary of electrophysiological effects. The concentration of oxyhemoglobin (OxyHb) was 5 μM and for methemoglobin (MetHb) it was 100 μM. Other agents were catalase (300 U/ml), superoxide dismutase (SOD, 170 U/ml), xanthine (X, 1 mM), xanthine oxidase (XO, 10 U/l), H_2O_2 (100 μM), $CuCl_2$ (100 μM). Ascorbic acid (100 μM) was added with the $CuCl_2$. Numbers in parentheses give the number of cells affected over the total number of cells assessed, if different. The treatments which eliminated potassium currents all initially increased them. Treatments which appeared to eliminate the potassium current may simply have resulted in conditions in which they could not be measured. An asterisk (*) denotes significance at the 0.05 level.

Table II-2. Viability of isolated smooth muscle cells was assayed by fluorescein diacetate uptake. Fluorescent cells were alive. Agents were OxyHb (oxyhemoglobin), MetHb (Methemoglobin), DMSO (dimethyl sulfoxide), SOD (superoxide dismutase), H.I. SOD (heat-inactivated superoxide dismutase), catalase, H_2O_2, Fe^{2+} (as unbuffered $FeCl_2$), xanthine and xanthine oxidase. An asterisk denotes $P < 0.05$.

	Fluorescence			
Treatment	**Yes**	**No**	**Total**	**% Viable**
Control	118	32	150	79
10^{-4} M OxyHb	61	89	150	41*
10^{-4} M MetHb	143	57	200	72
Control	83	17	100	83
10^{-7} M OxyHb	81	19	100	81
10^{-6} M OxyHb	70	30	100	70*
10^{-5} M OxyHb	39	36	75	52*
10^{-4} M OxyHb	43	57	100	43*
Control	167	33	200	83
10^{-4} M OxyHb	122	78	200	61
OxyHb + 1 U/ml SOD	135	65	200	68
OxyHb + 10 U/ml SOD	138	62	200	69
OxyHb + 100 U/ml SOD	147	53	200	74*
OxyHb + 1000 U/ml SOD	146	54	200	73*
10^{-4} M OxyHb	60	40	100	60
OxyHb + 170 U/ml H.I. SOD	64	36	100	64
Control	84	16	100	84
10^{-4} M OxyHb	58	42	100	58
OxyHb + 300 U/ml Catalase	76	24	100	76*
OxyHb + Catalase + 170 U/ml SOD	81	19	100	81*
Control	79	21	100	79
500 µM H_2O_2	34	16	50	68
H_2O_2 + 1 mM Fe^{2+}	29	71	100	29*
H_2O_2 + Fe^{2+} + 0.5% DMSO	97	53	150	65*
10^{-4} M OxyHb	40	60	100	40
OxyHb + 0.5% DMSO	69	31	100	69*
1 mM Xanthine	84	16	100	84
10 U/l Xanthine oxidase	80	20	100	80
Xanthine + xanthine oxidase	80	20	100	80

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CHAPTER III.

EFFECT OF BILIRUBIN ON ELECTRICAL PROPERTIES OF CEREBROVASCULAR SMOOTH MUSCLE CELLS

INTRODUCTION

About 30,000 cases of subarachnoid hemorrhage in North America each year result from rupture of intracranial aneurysms[9]. Angiographic evidence of cerebral vasospasm develops 5-7 days after aneurysm rupture in most surviving patients[3]. Delayed ischemic deficits and death are the potential consequences of vasospasm. Identification of the principal spasmogen in a simplified preparation could lead to the identification of the mechanism by which the spasmogen works and the design of a rational therapeutic strategy. This study was undertaken to compare the effects of bilirubin and oxyhemoglobin on the electrical properties of one such model system consisting of isolated cerebral arterial smooth muscle cells. The pathogenesis of vasospasm is poorly understood, but evidence implicates breakdown products of red blood cells in the clot. Oxyhemoglobin[1,15,19] and bilirubin[1,12] levels in cerebrospinal fluid parallel the clinical course of vasospasm. We have previously shown that oxyhemoglobin produces dramatic damage to isolated cerebral arterial smooth muscle cells[17]. At 1-10 μM, oxyhemoglobin causes these cells to contract within 1-2 min. Electrophysiologically, one observes a large increase in a potassium conductance which can be mimicked by raising the intracellular calcium by exposure to caffeine or by addition of calcium to the patch pipette. Longer exposure to oxyhemoglobin results in further contraction and the appearance of membrane blebs. Electrophysiologically, this later stage is marked by the appearance of a large leakage conductance. Cells in

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A version of this chapter has been submitted for publication. Stockbridge, Maljkovic & Weir 1991. *Neurosurgery*.

this state have lost the ability to concentrate fluorescein diacetate. Several lines of evidence suggest that oxyhemoglobin's deleterious effects are mediated by free radical reactions[14,16,17,20].

Bilirubin has detergent-like properties[2] and constricts intact cerebral arteries[4,5] and cerebral arterial segments denuded of endothelium[13]. In this study, the effects of bilirubin and oxyhemoglobin were compared in morphological and electrophysiological experiments on smooth muscle cells isolated from the rat basilar artery.

MATERIALS AND METHODS

Female Sprague-Dawley rats two months old were anesthetized with halothane and decapitated. The basilar arteries were removed to a solution consisting of (mM) NaCl 130, KCl 5, $CaCl_2$ 0.8, $MgCl_2$ 1.3, glucose 5, HEPES 10, plus penicillin (100 U/ml) and streptomycin (0.1 g/l) at pH 7.4. Arteries cleaned of connective tissue and small side branches were transferred to a medium in which the $CaCl_2$ was reduced to 0.2 mM and to which were added collagenase (Type II, 0.5 g/l), elastase (0.5 g/l), hyaluronidase (Type IV-S, 0.5 g/l) and deoxyribonuclease I (0.1 g/l). The arteries were cut into 0.2 mm wide rings and incubated for 1 hour at room temperature. The rings were transferred to 0.2 mM calcium saline containing trypsin inhibitor (0.5 g/l) and deoxyribonuclease I (0.1 g/l) and gently triturated. Cells were plated on glass cover slips and stored at 4°C in 0.8 mM calcium saline and essentially fatty acid free bovine serum albumin (2 g/l). Cells were used up to 48 hours after plating.

Whole cell membrane currents were recorded by the patch electrode voltage clamp technique[8] using an Axopatch 1C patch clamp amplifier (Axon Instruments). The patch pipettes had resistances of 1-4 MΩ. The input resistance was determined from small hyperpolarizing voltage steps and was 1-20 GΩ in normal cells. Series resistance compensation was not employed. As is conventional, outward current is positive. No leakage correction was applied.

For electrical recording, the bath solution contained (mM) NaCl 130, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1.2, glucose 5.2, HEPES 10, adjusted to pH 7.4 with NaOH. The pipette solution contained KCl 139, $MgCl_2$ 0.5, EGTA 2, glucose 10, HEPES 10, pH adjusted to 7.4 with KOH. All chemicals were obtained from Sigma.

For each experiment, 1 ml of a fresh colloidal stock suspension of bilirubin was prepared as 1 g/l in 1N NaOH. HCl (0.02 ml 1N) was added slowly followed by pH 7.4 sodium phosphate buffer (1 ml, 67 mM). The final bilirubin concentration was determined spectrophotometrically[10,21], but, as discussed, much of the 'nominal' concentration reported here was not dissolved bilirubin. The degradation of bilirubin in such a solution follows a complex multi-path course[11,22]. The principal constituent after prolonged incubation cannot be assumed to be bilirubin IXα, as it is in the beginning.

Rat oxyhemoglobin (Sigma) was prepared as a 1 mM stock solution in 150 mM NaCl, reduced by 10 mM $Na_2S_2O_4$. The reducing agent (which was without effect) was removed by dialysis.

RESULTS

Relaxed, isolated, phase bright cerebrovascular smooth muscle cells were studied. Such cells were previously shown to stain positively for α-actin and to constrict in response to elevated KCl, serotonin, angiotensin II and prostaglandins $F_{2\alpha}$ and E_1[17].

Because of rundown of outward currents and the limited lifetime of individual patch clamp recordings, three paradigms were employed to look for the effects of bilirubin over a time course of up to ~20 min, up to an hour and over several hours.

Isolated cells under whole cell patch clamp in the normal bath solution were voltage-clamped to a holding potential of -60 mV and subjected to 150 ms steps to potentials between -100 mV and +110 mV. The peak current obtained from such a series of records was plotted as a function of the voltage clamp command potential. Three such curves appear in figure 1. The outward current was blocked by substitution of Cs^+ for K^+ in the pipette. The initial maximum amplitude of the outward current varied from a few hundred pA to 1-2 nA. From experiments in which the intracellular calcium concentration was well buffered or in which tetraethylammonium chloride was added to the bath to block the calcium-dependent potassium current or in which procaine or strychnine in the bath were used to block the delayed rectifier potassium conductance, we conclude that the delayed rectifier contributes the few hundred pA seen in all recordings and the remaining variation reflects variation in the intracellular calcium concentration. Experiments with a high pipette calcium concentration or with caffeine in the bath showed that these cells can produce calcium-dependent potassium currents amounting to 2-4 nA or greater.

Under control conditions, these cells rarely showed net inward current (but see figure 4). The curve shown in open circles in figure 1was obtained immediately prior to the addition of 100 μM bilirubin to the bath. The other two sets of records were obtained beginning 1 min (solid squares) and 25 min (solid triangles) later. Over this time span, currents recorded were usually fairly stable and there was no apparent effect of bilirubin on either the outward current or the leakage current.

Over a longer time course, there was a tendency for the amplitude of potassium currents to decrease, as shown in figure 2. The times indicated were measured from the beginning of the whole cell recording. This 'rundown' phenomenon occurs in other preparations and is thought to result from displacement of some important soluble cytosolic component by the contents of the patch pipette.

Patch recordings obtained in the presence of bilirubin showed a similar time course for the rundown of the potassium current. Figure 3A shows a set of six voltage steps and current recordings obtained at 1 min, 20 min and 80 min following addition of bilirubin to the bath. The current-voltage plots from this data appear in figure 3B.

Cells maintained for 11 h in the presence or absence of bilirubin (100 μM) were compared (figure 4). In this case, the voltage clamp steps and the current records are shown in insets. Both of these cells show some net inward current. No ion substitution experiments were performed on these cells, but in other experiments, inward currents could be blocked by bath application of Cd^{2+}, Ni^{2+} or Co^{2+}, but not by substitution of $choline^+$ for Na^+ or by substitution of Ba^{2+} or Sr^{2+} for Ca^{2+}, so we presume this inward current is carried by calcium ions. The cell which was exposed to bilirubin had a somewhat larger calcium component, and perhaps as a consequence, a somewhat larger and steeper-rising potassium current. There was no consistent relationship observed between the magnitude of inward or outward currents and exposure to bilirubin.

Prolonged exposure, up to 8 h, to bilirubin (100 μM) produced no morphological changes in the smooth muscle cells. Figure 5 shows pairs of pictures obtained shortly after dissociation and after 30 and 180 min at room temperature. Bilirubin was added to the right dish just after its 'time 0' photograph.

In contrast, cells exposed to oxyhemoglobin underwent morphological and electrical changes that were easily observable. Figure 6 shows current records obtained from a cell under normal conditions (first column) and with addition to the bath of 10 μM oxyhemoglobin for 30 s (middle column) and 2 min (right column). By 30 s, a large outward current developed. By 2 min, a large leakage current developed and most of the time-dependent current was gone. Coincident with the electrical changes, cells exposed to oxyhemoglobin (not just the cell being voltage-clamped) contracted and within several minutes developed blebs.

The lifetime of whole cell recordings was measured in all experiments. The end-point was the development of a leakage current greater than the time- and voltage-dependent current. For cells exposed to saturated solutions of bilirubin the lifetime was 40.7 ± 14.8 min (S.E.M.; n = 7). For cells in the normal medium, it was 45 ± 30 min (n = 2). For cells exposed to oxyhemoglobin (after initiation of whole-cell recording) it was 5.1 ± 1.5 min (n = 9). A pair-wise t-test established no difference between the lifetimes in control media and with bilirubin (P = 0.39), but exposure to oxyhemoglobin produced a significant reduction compared with either the control or bilirubin-containing media (both P = 0.02).

DISCUSSION

This study compared the effects of bilirubin and oxyhemoglobin on the electrophysiological properties of isolated cerebrovascular smooth muscle cells.

Bilirubin IXα is an intermediate step in the breakdown of the heme ring in hemoglobin. This compound is unstable in solution[22] as a result of auto-oxidation, aggregation and surface adsorption. At physiological pH, bilirubin precipitates from a 25 μM aqueous solution[11]. Spectrophotometric assays for bilirubin[7,10,21] used in this study did not distinguish among dissolved bilirubin, some products of bilirubin auto-oxidation, and perhaps colloidally dispersed bilirubin that passed a 0.1 μm filter. The actual free bilirubin concentration in contact with our cells cannot, therefore, be determined, but was probably about 0.1 μM[10]. However, considerably more bilirubin in some undissolved state was in contact with the cells. No attempt was made to increase the amount of dissolved or suspended bilirubin, e.g. by using dimethyl sulfoxide or albumin, because no such mechanism is present to make bilirubin more soluble in cerebrospinal fluid.

Bilirubin is believed to be potentially cytotoxic because of detergent properties[2], but there is evidence that albumin-bound bilirubin may act as a physiological anti-oxidant of lipid peroxidation[6,18].

Duff *et al.*[4,5] have studied the possible role of bilirubin in the pathogenesis of cerebral vasospasm following subarachnoid hemorrhage. They exposed cat and baboon cerebral arteries to a saturated bilirubin solution and found a significant reduction in vessel diameter developed over 4 h. Miao & Lee[13] exposed pig internal carotid and dog basilar arteries *in vitro* to bilirubin and found endothelium-independent contractions which developed over a period of minutes. Upon washout of bilirubin, the vessels relaxed but had potentiation of α_2 adrenoreceptor-mediated constriction.

In the present study, smooth muscle cells isolated from the rat basilar artery were exposed to saturated solutions of bilirubin for periods up to 11 h. This treatment failed to produce significant contraction and there was no rise in calcium-activated potassium current. Both of these findings suggest there was no significant rise in intracellular free calcium. Cells retained a normal permeability barrier in bilirubin, which would not have been expected had bilirubin acted like a detergent.

Buffering the calcium in the pipette at elevated levels induced a large outward current in these smooth muscle cells. This current is similar to calcium-activated currents described in many other cells. The contraction of these smooth muscle cells and the appearance of a large outward current produced by oxyhemoglobin suggests that oxyhemoglobin induced a rise in intracellular calcium. This rise in intracellular calcium may have quickly reached levels which became damaging, evidenced by the appearance of membrane blebs and a loss of the normal, ion-selective permeability barrier of the cell membrane.

These experiments do not rule out a contribution of bilirubin to the pathogenesis of cerebral vasospasm. However, effects others have reported to occur over a time course of hours are unlikely to be direct effects on smooth muscle cells. In contrast, oxyhemoglobin does have direct and deleterious effects on smooth muscle cells. Work on the mechanism by which oxyhemoglobin produces these effects is in progress.

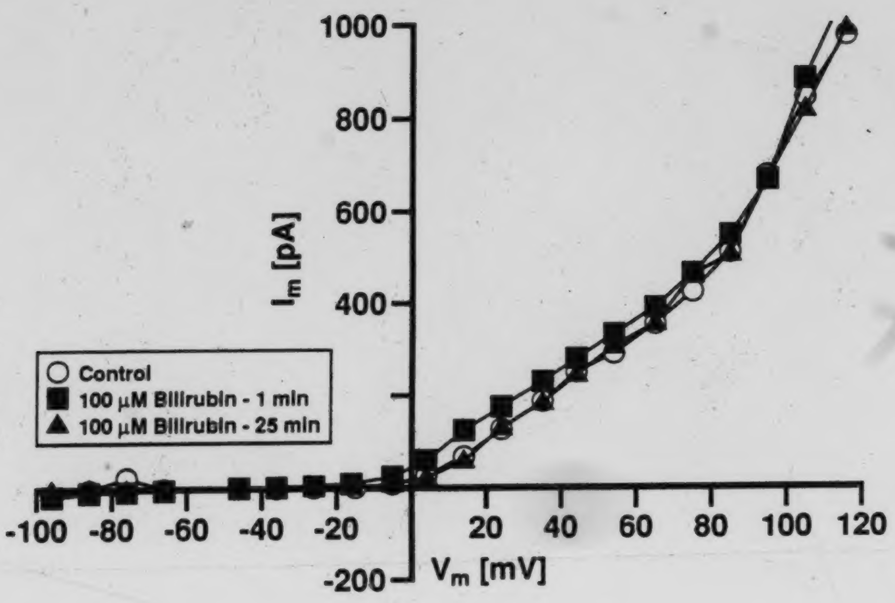


Figure III-1. Brief exposure to bilirubin had no effect on the electrophysiological properties of smooth muscle cells. A single basilar artery smooth muscle cell was studied by the whole cell patch clamp technique. The cell was held at -60 mV and stepped for 150 ms to potentials from -100 to +110 mV. The peak current elicited was plotted as a function of the test potential. Outward currents (carried by potassium ions) and depolarizing potentials (relative to the bath) are positive. A set of records was obtained in the normal bath medium (open circles) and then bilirubin (nominally 100 µM) was added to the bath. Similar data sets were collected after 1 min (solid squares) and 25 min (solid triangles).

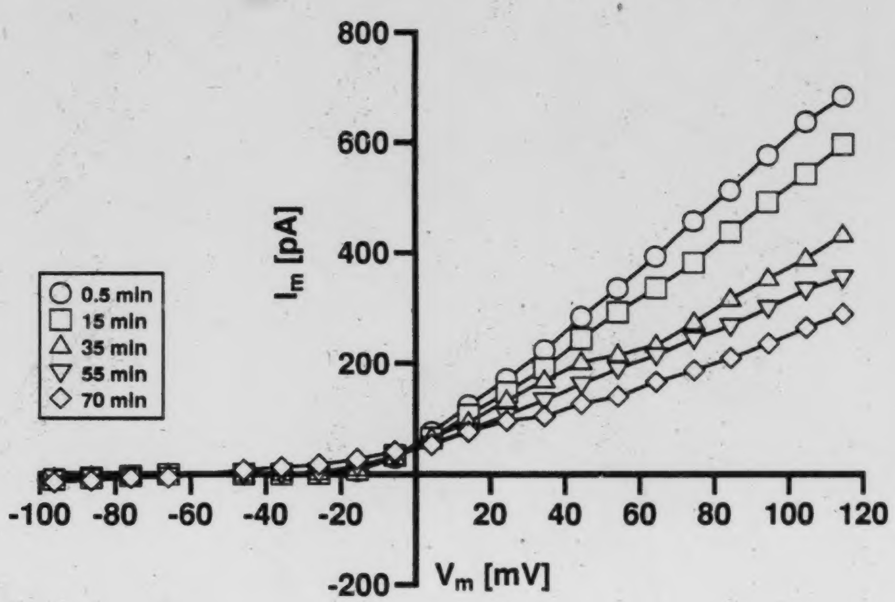
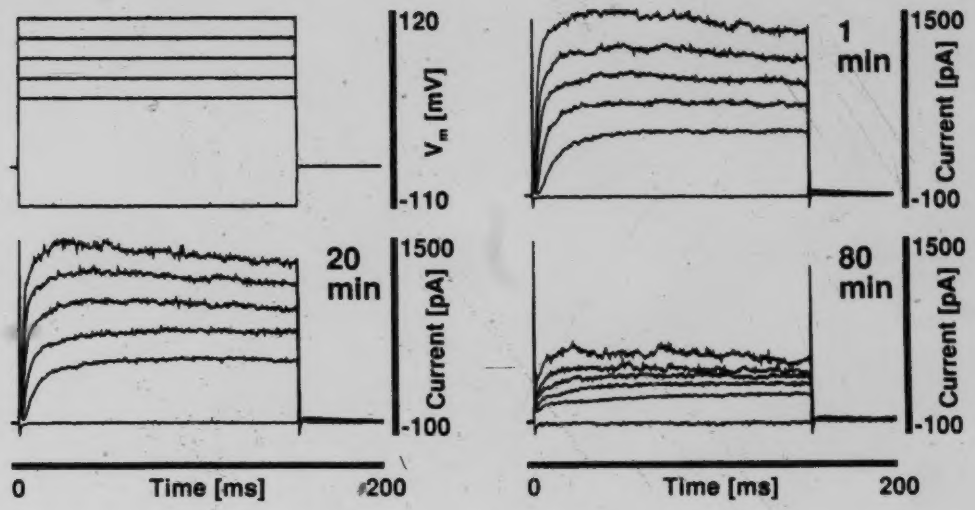


Figure III-2. A smooth muscle cell under whole cell patch clamp conditions in the control medium experienced a decrease in the magnitude of the outward current. In this case, about half of the current disappeared by 55 min. The decrease in current amplitude is believed to reflect the dependence of the delayed rectifier type of potassium conductance on some soluble cytosolic component that is lost through dialysis of the pipette solution into the much smaller cell volume.

Figure III-3. A smooth muscle cell exposed to bilirubin (nominally 500 μM) while under whole cell patch clamp showed a reduction in the amplitude of the outward current with a time course similar to that of a cell in the control solution. **A.** The top left panel shows the voltage clamp protocol (6 overlaid traces). The other three panels show the corresponding current records obtained after 1 min (top right), 20 min (bottom left) and 80 min (bottom right). **B.** Peak current vs. voltage clamp command potential for the cell whose current records appear in Figure 3A. Half of the current amplitude was left after about 50 min.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-110
1
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Current [pA]
-100
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min
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-100
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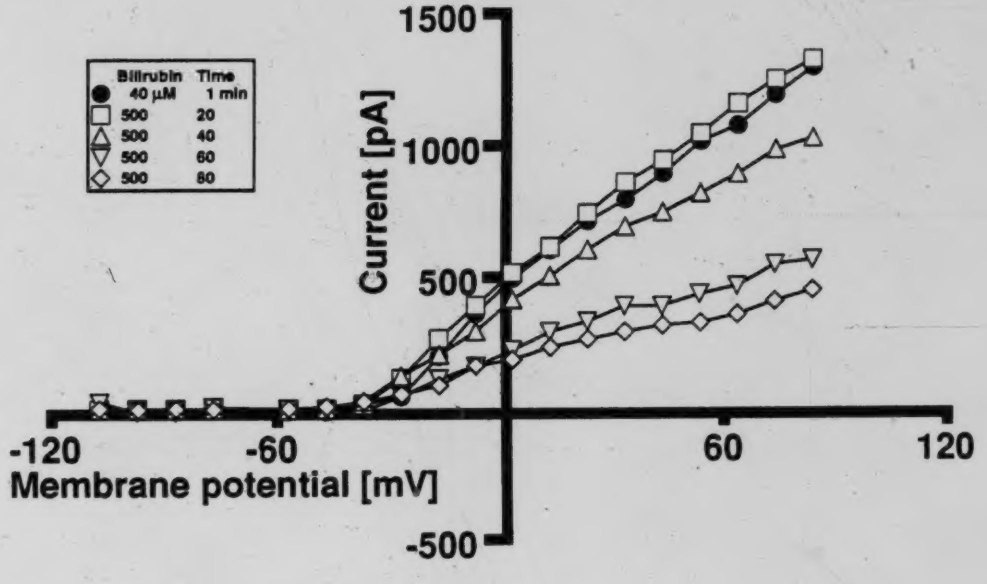
min
1500
Current [pA]
-100
0
Time [ms]
200
0
Time [ms]
200


Bilirubin Time
● 40 µM 1 min
□ 500 20
△ 500 40
▽ 500 60
◇ 500 80
1500
1000
500
-500
Current [pA]
-120
-60
60
120
Membrane potential [mV]

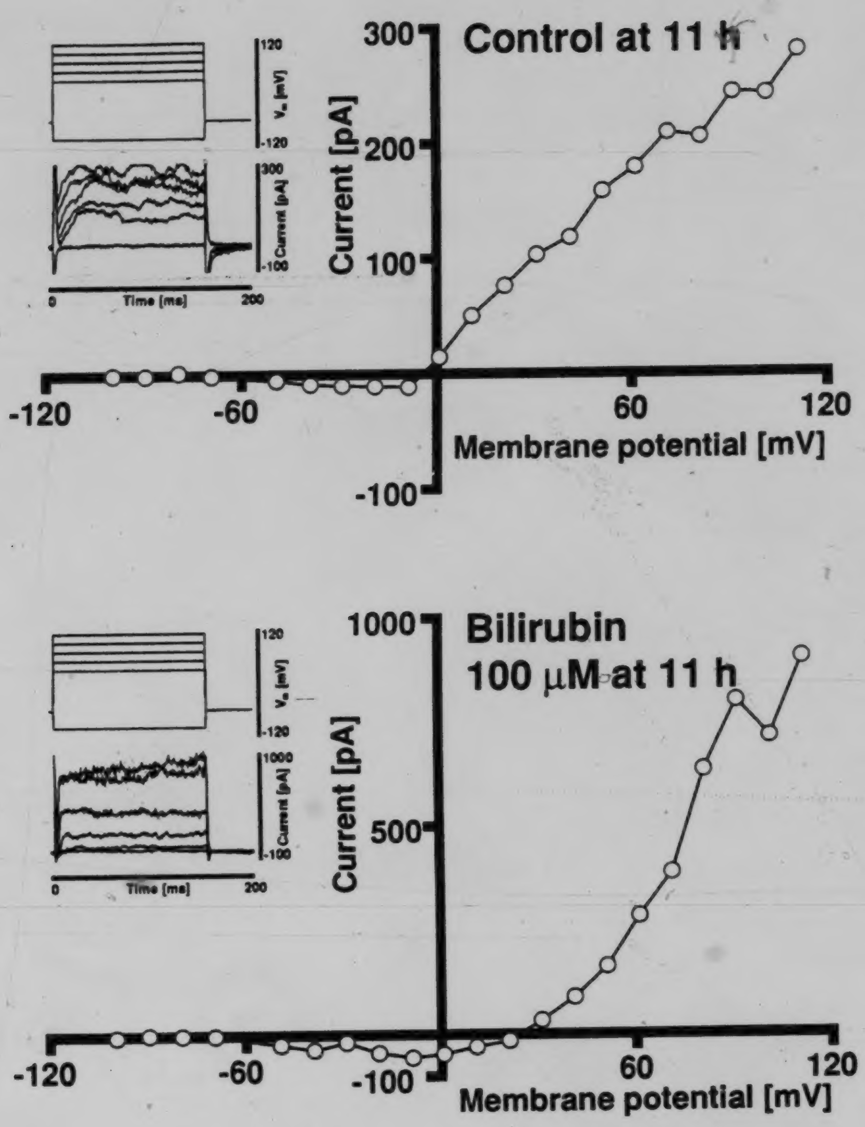


Figure III-4. Smooth muscle cells were preincubated in the normal bath solution (top) or bilirubin (nominally 100 µM; bottom) for up to 11 h before whole cell patch clamp recording. The voltage clamp steps and the current records are shown in insets. Both cells showed evidence of net inward (negative) current at potentials between -60 mV and 0-20 mV. This current was most likely mediated by calcium channels. The difference in the magnitude of the currents is probably not the result of bilirubin.

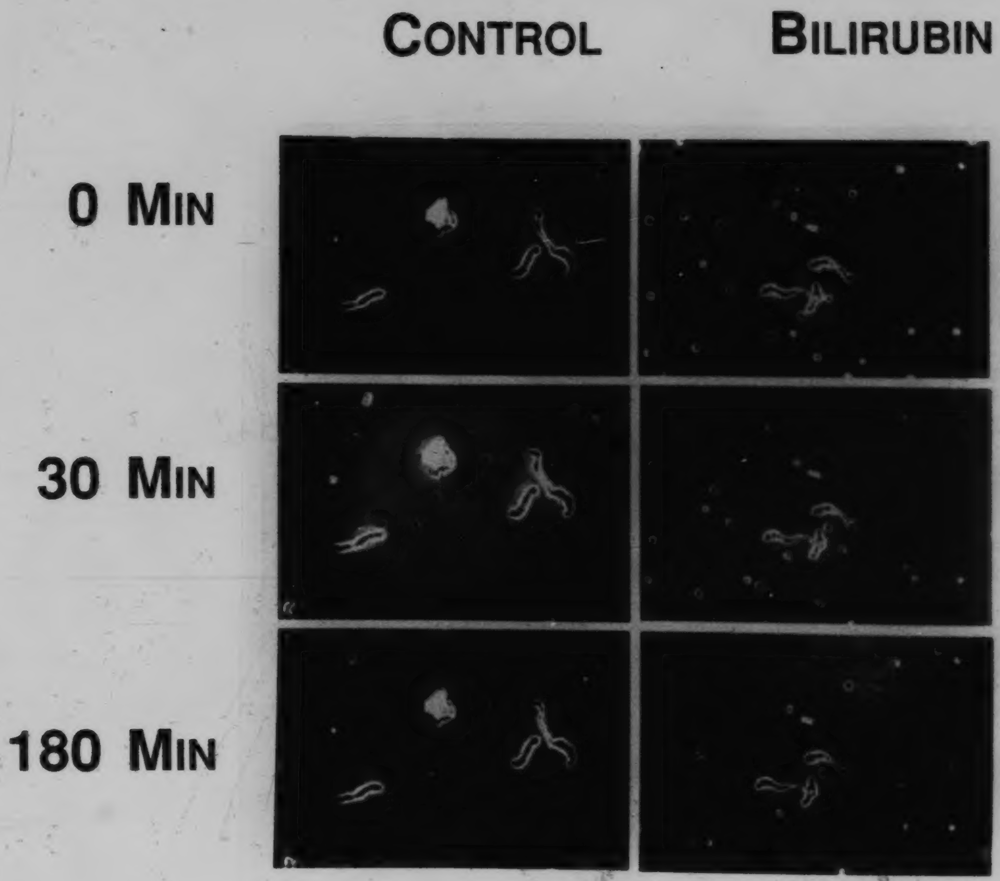
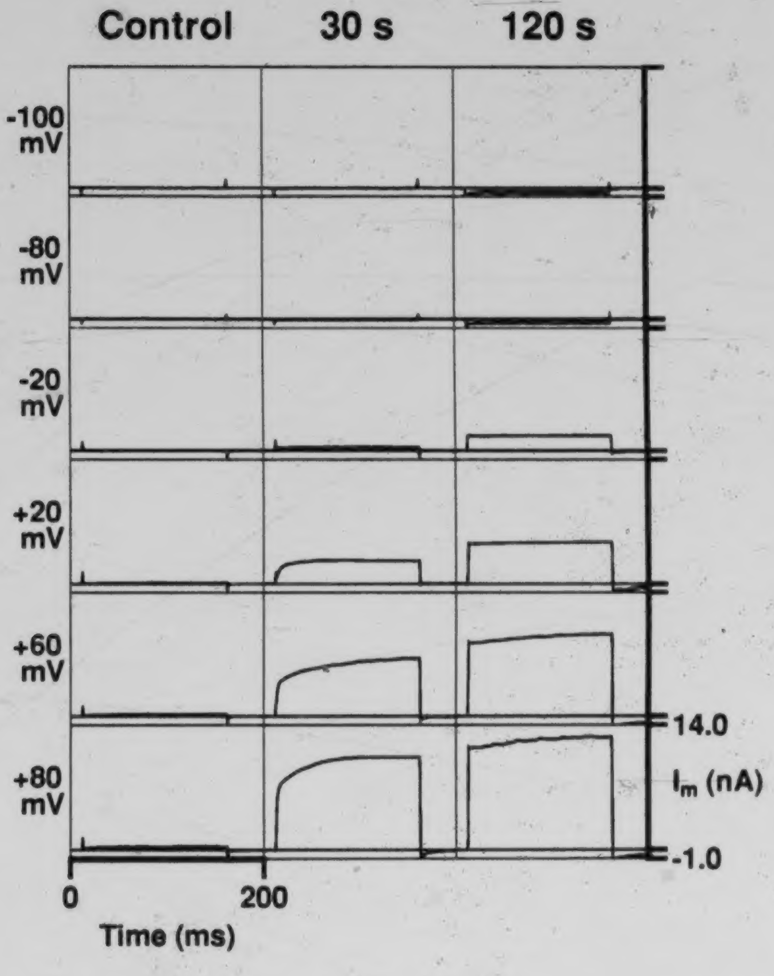


Figure III-5. Smooth muscle cells were maintained in covered dishes at room temperature on the microscope stage in either the normal bath solution or the bath solution plus 100 μM bilirubin. Cells were neither maximally relaxed nor maximally contracted at the beginning of the experiment. Neither group of cells changed morphology during 3 hours of observation.

Figure III-6. The first column shows current records from a smooth muscle cell in the normal medium obtained in response to voltage steps to the potentials indicated in the left margin. The second column shows current recordings obtained in the same cell for the same voltage steps beginning 30 s after application of 10 μM oxyhemoglobin. A large time- and voltage-dependent outward current has appeared. By two min after addition of oxyhemoglobin to the bath (column three), a time- and voltage-independent conductance has appeared and the time- and voltage-dependent component is substantially reduced. The cell from which the recording was made and other cells in the dish were contracted and showed membrane blebs.


Control
30 s
120 s
-100 mV
-80 mV
-20 mV
+20 mV
+60 mV
+80 mV
14.0
I_m (nA)
-1.0
0
200
Time (ms)

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CHAPTER IV.
DISCUSSION, CONCLUSIONS, AND RECOMMENDATIONS

This is not an end.
It is not even the beginning of the end.
But it is, perhaps, the end of the beginning.
Winston Churchill (London, 10th November 1942)

DISCUSSION

Although the pathogenesis of late cerebral vasospasm following subarachnoid hemorrhage due to rupture of an intracranial aneurysm still remains unknown, it seems highly likely that the cascade of events is initiated by lysis of erythrocytes from blood clot.

We have developed a model system in which to study the electrophysiologic properties of single smooth-muscle cells from cerebral blood vessels. To do this, first we had to establish optimum conditions for isolating the single cells from rat cerebral arteries and plate them as a primary culture. Modification of the procedure published by Worley et al. (1) was our starting point for developing the model. As the use of enzymes to isolate skeletal muscle fibers does not alter membrane conductances (2), we used enzymes and light mechanical force to isolate cerebrovascular smooth-muscle cells. Nevertheless, we were concerned that some membrane components may be damaged by the dissociation procedure and thus had to verify that the cells remained viable and retained their morphologic and physiologic properties. First, the retention of fluorescein diacetate (FDA) dye was tested as a measure of viability (3), on the principle that live cells incubated with membrane-permeable non-fluorescent FDA enzymatically cleave this to the charged molecules of less-permeable fluorescein with fluorescence properties; thus, viable cells are highly fluorescent and non-viable cells are non-fluorescent. Second, we demonstrated that the smooth-muscle cells were spindle-shaped and stained positive for α-actin, a specific marker for smooth-muscle cells (4). Third, the cells contracted upon exposure to caffeine, angiotensin II, $PGF_{2\alpha}$, and high extracellular concentrations of potassium.

After verification that the isolated smooth-muscle cells from rat basilar artery were viable and had retained their morphologic and physiologic properties, we sought to determine whether the patch-clamp technique could be applied to the study of ion channels in these cells (5), as it had been established for other vascular smooth-muscle cells in voltage-clamp studies with the whole-cell and single-channel patch clamp technique (6,7). Control of the voltage for recordings made with a patch-clamp is superior to that for recordings made with conventional microelectrode techniques in intact vessels, because the cells are single, small, not electronically coupled, and no longer surrounded or affected by connective tissue.

In our laboratory Dr JA Steele was characterizing the membrane properties of isolated smooth-muscle cells, using whole-cell and single-channel voltage-clamp techniques. Ion channels were characterized on the basis of their ion selectivity, voltage dependency, kinetics and pharmacology. The high- and low-threshold calcium currents, as well as the delayed-rectifier, calcium-activated, spontaneous transient, and inward-rectifier potassium currents were 'fingerprinted' in this way.

Having developed this model system to study the pathophysiologic basis of cerebral vasospasm following SAH, we assessed the effects and mechanism of action of blood breakdown products (especially oxyhemoglobin and bilirubin) that seem likely candidates in the etiology of late cerebral vasospasm.

Oxyhemoglobin, a breakdown product of blood induces contraction of cerebral and peripheral blood vessels (8) and has multiple effects on single cerebrovascular smooth-muscle cells (9). A rise in intracellular free calcium is necessary to initiate contraction of cerebrovascular smooth muscle (10), but the mechanism by which oxyhemoglobin causes this contraction is still not clear. It was interesting that oxyhemoglobin had no detectable effect on neuroblastoma cells (Ch. II, Fig. 2) which have no intracellular calcium stores, indicating that the effects of oxyhemoglobin on cerebrovascular smooth muscle may result from an increase of intracellular free calcium. Smooth-muscle cells developed membrane blebs within 5 minutes after exposure to oxyhemoglobin, indicating severe, irreversible damage to the cell membrane.

When a whole-cell voltage clamp was in place, single smooth-muscle cells evidenced greatly increased outward currents and a decreased input resistance when oxyhemoglobin was applied. Tomita (11), who studied ionic channels in smooth muscle with patch-clamp methods, reported that vascular smooth-muscle cells have a high density of calcium-activated potassium channels. Caffeine increases the concentration of free intracellular calcium by releasing it from the sarcoplasmic reticulum (12); in the present study, it increased the macroscopic outward currents by releasing calcium from intracellular stores and by opening calcium-activated potassium channels for a longer time (13). Oxyhemoglobin greatly increased the outward currents. If calcium-activated potassium channels are an indicator of the intracellular concentration of free calcium, and if oxyhemoglobin increases the intracellular free calcium, the assumption can be made that cell contraction, membrane blebs, increased outward currents, increased membrane conductance and cell death contribute directly to an increased concentration of intracellular free calcium by releasing calcium from intracellular stores.

Vollrath et al. (14) reported that oxyhemoglobin can act on phospholipase C and stimulate the hydrolysis of phosphatidylinositol 4,5-bisphosphate, with consequent elevation of inositol 1,4,5-trisphosphate - which can act as a second messenger, releasing calcium from the sarcoplasmic reticulum of vascular smooth-muscle cells. This finding supports the hypothesis that oxyhemoglobin contracts vascular smooth muscle by producing inositol trisphosphate, and thus releasing calcium from intracellular stores. Rat aortic smooth-muscle cells (A_7r_5) were used in Vollrath et al.'s experiments, because of the finding in smooth-muscle cells from rat aorta (15) that oxyhemoglobin induced changes closely resembling those seen in cerebrovascular smooth-muscle cells after subarachnoid hemorrhage.

Neomycin (5 mM), an antagonist of phospholipase C, delayed and diminished the pharmacologic responses to oxyhemoglobin, by reducing the oxyhemoglobin-induced elevation of inositol trisphosphate. Neomycin also reduced pharmacologic responses of smooth-muscle cells to norepinephrine, thrombin, and potassium chloride, by depressing production of the second messenger. When neomycin is present, potassium chloride can still induce contraction of smooth-muscle cell by the promoting the entry of calcium through dihydropyridine-sensitive low-threshold calcium channels.

Oxyhemoglobin increases levels of inositol trisphosphate, by activating phospholipase C through a G-protein sensitive to pertussis toxin, but this effect is brief and values return to normal within 10 minutes when oxyhemoglobin is applied.

In all of the above experiments, the external medium contained calcium chloride (1.5-1.8 mM). When all traces of calcium were removed from the bathing medium, oxyhemoglobin had no damaging effect on cerebrovascular smooth-muscle cells: calcium-activated potassium current did not increase, and input resistance did not decrease, for up to 90 minutes (unpublished data in experiments with patch-clamp technique).

If oxyhemoglobin induced smooth-muscle cell contraction only by releasing free calcium from intracellular stores, the concentration of extracellular calcium would make no difference. In fact, however, this does affect the degree of contraction (unpublished data).

Chronic cerebral vasospasm after SAH seems to result from a cascade of events initiated by lysis of blood clot and the release of oxyhemoglobin. Upon spontaneous oxidation of oxyhemoglobin to methemoglobin, superoxide anion is released (16). Superoxide is converted by superoxide dismutase to hydrogen peroxide, which under normal conditions is inactivated by catalase or glutathione peroxidase, but these defense mechanisms are very poor in brain tissue and almost undetectable in the CSF of the higher mammals, including man. Pure hemoglobin can be degraded by excess hydrogen peroxide to release 'catalytic' iron ions from the heme ring (17). In addition, when oxyhemoglobin comes in contact with even tiny traces of hydrogen peroxide it generates a powerful oxidizing species (probably ferryl) that can stimulate lipid peroxidation (18). All these active free radicals initiate reactions, such as peroxidation of polyunsaturated fatty acids in the biomembrane, and produce fatty-acid free radicals - which readily combine with oxygen to become lipid peroxide, which in turn produces more free radicals and thereby starting a chain reaction of lipid peroxidation (19). Lipid hydroperoxides inhibit synthesis of prostacyclin, the most potent vasodilator, and activate cyclo-oxygenase and C12-lipoxygenase within the clot. Products of C12-lipoxygenase activate C5-lipoxygenase in leukocytes and the arterial wall. Prostaglandins such as PGH_2 and thromboxane A_2 are the cyclo-oxygenase products of arachidonic-acid metabolism, and leukotrienes are the products of 5-lipoxygenase. Both prostaglandins and leukotrienes are potent vasoconstrictors; they are thought play an important role in cerebral vasospasm.

The data presented in Chapter II support the hypothesis that the generation of free radicals by oxyhemoglobin is responsible for its effects on smooth muscle. Direct application of methemoglobin, which does not generate free radicals, had no effect on smooth-muscle cell contraction, greatly increased outward currents, and decreased input resistance. Also, DMSO and catalase, both of which eliminate hydroxyl radicals, protected against oxyhemoglobin.

An excess of hydrogen peroxide or superoxide anion, produced by xanthine plus xanthine oxidase, increased calcium-activated potassium currents but had no effects on input resistance or cell viability. This suggests that when oxyhemoglobin comes in contact with traces of hydroxy peroxide it generates a powerful oxidizing species (probably 'ferryl') that can stimulate lipid peroxidation, but appears to be more reactive than hydroxyl radicals and less affected by scavengers such as salicylate, phenylalanine, and arginine. It appears that only this powerful oxidizing species is produced when oxyhemoglobin is applied to smooth-muscle cells from rat basilar artery (Ch. II). On the other hand, the ability of oxyhemoglobin to generate both this reactive species and hydroxyl radicals in the presence of hydroxy peroxide may account for the development of cerebral vasospasm following SAH.

In Chapter III we compared the effects and mechanism of action of bilirubin and oxyhemoglobin on the electrophysiologic properties of isolated cerebral arterial smooth-muscle cells.Bilirubin IXα is a hemoglobin breakdown product with detergent-like properties. It is unstable in solution as a result of several distinguishable processes: auto-oxidation, precipitation-aggregation and surface adsorption. At pH 7.4-8.8 bilirubin precipitates from 2.5 x 10^{-5} M solutions and adsorbs to the walls of a glass container. In addition, and bilirubin undergoes an oxygen-dependent isomerization and also has a tendency to form colloidal or insoluble aggregates. (20). The solubility of bilirubin in 0.1 M phosphate buffer at pH 7.4 appears to be less than 0.005 mg/100 ml (21). Therefore, the actual concentration of free bilirubin in contact with our cells cannot be determined, but was probably 10^{-7} M. However, a lot of bilirubin in suspension was in contact with smooth-muscle cells.

Albumin-bound bilirubin appears to be an important plasma antioxidant of lipid peroxidation (22), but bilirubin is also believed to be potentially cytotoxic because of detergent-like activity (23).

The effects of topical applications of bilirubin to basilar arteries were studied in cats and baboons in vivo (24,25). The major finding of this study was that saturated bilirubin solution induced severe constriction and pathologic changes when applied to cat or baboon basilar arteries. Miao and Lee (26) demonstrated that bilirubin induced dose-dependent contractions of pig and cat internal carotid arteries and dog basilar arteries in vitro.

In our study, smooth-muscle cells isolated from the rat basilar artery were exposed to saturated solutions of bilirubin for periods up to 11 h, but bilirubin did not induce significant cell contraction and did not increase calcium-activated potassium current during that time suggesting that there was no significant rise in intracellular calcium. Also, bilirubin did not act like a detergent; cells retained their normal permeability barrier even after 11 h of exposure to bilirubin (Fig. III-4).

These experiments do not rule out a contribution of bilirubin in the development of cerebral vasospasm following the SAH. Effects others have reported to occur over a long time are unlikely to be direct effects on smooth-muscle cells and the mechanism by which bilirubin causes these vascular changes has not been clarified. In contrast, oxyhemoglobin induces direct and irreversible damage on cerebrovascular smooth-muscle cells.

CONCLUSIONS

In summary, the most important findings in this investigation are:

1. The development of a model system in which to study the electrophysiologic properties of single smooth-muscle cells from cerebral blood vessels.

2. The verification that the isolated smooth-muscle cells from rat basilar artery were viable and had retained their morphologic and physiological properties.

3. The assessment of the effects of oxyhemoglobin on properties of cerebrovascular smooth-muscle cells:

    a. cell contraction

    b. the appearance of membrane blebs

    c. an increase in outward potassium currents

    d. a decrease in the membrane resistance

    e. cell death

4. The assessment of the mechanism of action of oxyhemoglobin.

5. The assessment of the effects and mechanism of action of other breakdown products of blood (methemoglobin and bilirubin) on electrical properties of cerebrovascular smooth-muscle cells.

RECOMMENDATIONS

Future investigations should include:

1. Confirmation of the rise of intracellular calcium: During the early stages of cell damage by oxyhemoglobin, hydroxyl radicals or 'ferryl' radicals there is a rise in intracellular calcium, but contraction of the cell and a large increase in the calcium-activated potassium current are only the circumstantial evidences of this rise. We would like to confirm these finding with Fura-2-AM, a fluorescent dye that determines the amount of free intracellular calcium. We propose to load smooth-muscle cells from rat basilar artery for a short time with Fura-2 dye, add different concentrations of oxyhemoglobin (0.1-10 μM) to the bath and establish a steady-state of intracellular calcium level or a slope for the accumulation of intracellular calcium.

2. Confirmation of relative contributions of intracellular and extracellular calcium pools: Do oxyhemoglobin and free radicals cause release of calcium from intracellular stores, or do they insult cell membranes? More whole-cell patch-clamp experiments should be designed to compare effects of oxyhemoglobin and other breakdown products from blood on isolated smooth-muscle cells in the normal bath (1.8 mM Ca^{2+}) and no calcium bath (and 2 mM EGTA). We could confirm these findings by loading cells with Fura-2 dye, when cells are in the normal calcium bath or in no calcium bath as above. If most of the calcium is released from intracellular stores, our effort should be in removal of intracellular free radicals and/or depletion of intracellular stores of calcium. If the cell membrane is leaky, then we should try to destroy extracellular free radicals.

3. Estimation of relative sensitivities to intracellular and extracellular free radicals: by measurement of the response of smooth-muscle cells to hydroxyl-radical-

generating system on either surface of the cell membrane. a.) The hydroxyl radical-generating system will be mixed in a dish and added to the bath of smooth-muscle cells at different time intervals and the whole-cell patch-clamp experiments will be performed to determine the base-line data that will be helpful in interpretation of data from experiments in which hydroxyl-radical-generating system is located intracellularly. b.) The calcium-activated potassium current and the leakage current will be monitored in isolated smooth-muscle cells that are exposed to hydroxyl-radical-generating-system from either surface of the cell membrane. The dose-response curves will be generated.

4. Determination of damage to lipid or ion channels by oxyhemoglobin: by study of calcium-activated potassium channels in cell-free membrane patches from isolated smooth-muscle cells, we will be able to determine if the membrane permeability increase comes from the lipid or ion-channel damage; under low calcium condition, the average number of calcium-activated potassium channels will be small and a rise in calcium will not be able to disrupt the cell membrane, but a damage to a lipid bilayer will show as an ungated pore.

5. Inhibition of oxyhemoglobin or free-radical generating system by indomethacin or iloprost using cell viability and morphometric analyses. Isolated cerebral arterial smooth-muscle cells will be divided in 3 dishes. One dish will receive oxyhemoglobin (10^{-5} M) and other 2 dishes will be pretreated with indomethacin (10^{-5} M) or iloprost (0.01-100 μg). Cell viability testing will be performed and cells compared.

6. Investigation of membrane properties of normal and vasospastic cerebral vascular smooth-muscle cells of primates and humans. Membrane properties of smooth-muscle cells from normal and vasospastic cerebral arteries will be compared. This will be done only occasionally when these arteries became available.

7. Screening of potentially useful nonspecific vasorelaxing drugs. Many nonspecific vasorelaxant drugs will be tried to see if they are effective in blocking the effects of oxyhemoglobin. If these drugs have known mechanism of action, they may provide some insight of the mechanism controlling cerebrovascular smooth-muscle tension.

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APPENDIX I.

A. ISOLATION OF SMOOTH MUSCLE CELLS

This procedure was developed in our laboratory to isolate vascular smooth-muscle cells from rat and primate cerebral arteries.

Female Sprague - Dawley rats 2 months old were anesthetized with halotane and decapitated below the base of the skull. The brainstem was exposed, removed with curved forceps, and placed in ice-cold solution consisting of (mM) NaCl 130, KCl 5, $CaCl_2$ 0.8, $MgCl_2$ 1.3, glucose 5, HEPES 10, plus penicillin (100 U/ml) and streptomycin (0.1 g/liter) at pH 7.4. Basilar arteries were cleaned of connective tissue and smaller branches, transferred to a medium in which the $CaCl_2$ was reduced to 0.2 mM, and to which were added (g/liter): collagenase (Type II, 0.5), elastase (0.5), hyaluronidase (Type IV-S, 0.5) and deoxyribonuclease I (0.1). The arteries were cut into rings 0.2 mm wide, and incubated for 1 hr at room temperature. The rings were gathered with a fire-polished Pasteur pipette, placed in medium containing 0.2 mM $CaCl_2$, trypsin inhibitor (0.5 g/liter), and deoxyribonuclease I (0.1 g/liter), and then gently triturated with a Pasteur pipette for up to 5 min in dishes with pieces of cover slip on the bottom. The cells were left for 10-15 min to attach to the cover slips; then the solution was slowly replaced with 0.8 mM calcium saline containing essentially fatty-acid-free bovine serum albumin (2 g/liter) and the cells were stored at 4^0C. After isolation the cells were kept in the refrigerator, to prevent division and differentiation, and were used within 48 hrs. Only a few cells were in each shallow culture dish, and they were adequately oxygenated by diffusion from room air.

The same isolation procedure was used to obtain smooth-muscle cells from primate middle cerebral arteries, but these isolated cells were not always usable.

Cells from rat basilar artery possess almost the same electrophysiologic properties as those from the middle cerebral artery of primates (unpublished data); this encouraged us to work mostly on smooth-muscle cells from rat basilar artery and to do comparative studies on middle cerebral arteries from primates only when these became available from other researchers using the primate model of vasospasm that most closely resembles the pathologic course in humans (1).

The present studies were of rat basilar artery, because of the angiographic evidence that vasospasm occurs in the large arteries.

B. SCREENING ASSAYS

CELL VIABILITY

Fluorescein diacetate (FDA) assay was used for testing cell viability. When incubated with membrane-permeable non-fluorescent FDA, live cells enzymatically cleave this molecule to a less-permeable fluorescein, producing very bright cells. Dead or leaky cells cannot cleave, or keep fluorescein inside the cell and thus do not fluoresce (2). As it is technically difficult to do enough patch-clamp experiments to reach a statistically significant conclusion for pharmacologic testing, cell viability was used instead.

MORPHOMETRIC ANALYSIS

Morphometric analysis was sometimes used to compare the effects of hemoglobin and bilirubin on contraction of single isolated smooth-muscle cells.

APPENDIX II.

CHARACTERIZATION OF RAT BASILAR ARTERY SMOOTH-MUSCLE CELLS AND THEIR IONIC CURRENTS

(Parallel studies by Dr Joy A. Steele in this laboratory)

Enzymatically dissociated smooth-muscle cells from rat basilar artery stained positive for α-actin, a specific marker for smooth-muscle cells (3), and contracted to KCl, caffeine, angiotensin II, $PGF_{2\alpha}$, PGE_1 and serotonin. As isolated cells are no longer attached to elastic connective tissue, when the contractive agent is removed they cannot relax completely.

Whole-cell ionic currents were recorded following the procedure of Hamill et al. (4) using an axopatch 1B or 1C patch-clamp amplifier (Axon Instruments, Burlingame, CA).

CALCIUM CURRENTS

Calcium currents were recorded when outward potassium currents were suppressed by a pipette solution containing (mM): 131.5 CsCl, 10 TEA-Cl, 2 EGTA and 10 HEPES. Bath solution contained (mM): 92 $BaCl_2$, 10 glucose, and 10 HEPES. Two different calcium currents were characterized in smooth cells of rat basilar artery.

THE HIGH-THRESHOLD CALCIUM CURRENT, which is similar to the 'classical' L-current recorded in other preparations, was activated by potentials above -20 mV, peaked at about +20 mV, and was sensitive to dihydropyridines, including nimodipine and Bay K 8644; its inactivation appeared not to be voltage-dependent. This current was recorded from most cells.

THE LOW-THRESHOLD CALCIUM CURRENT, which is similar to T-currents recorded in other preparations, was activated at potentials above -40 mV, peaked at about 0 mV, and was insensitive to dihydropyridines. Unlike T-currents, however it did not inactivate rapidly: its magnitude declined over the course of 10-20 minutes. This current was recorded in only 10% of cells.

POTASSIUM CURRENTS

Potassium currents were recorded with use of a pipette solution containing (mM): 134 KCl, 0.5 $MgCl_2$, 11 EGTA and 10 HEPES. The bath solution contained (mM): 130 NaCl, 5 KCl, 2 $CaCl_2$, 1.3 $MgCl_2$, 5 glucose and 10 HEPES.

DELAYED-RECTIFIER POTASSIUM CURRENT was activated by depolarizations above -35 mV and was blocked by external application of 1 mM of procaine or strychnine, or 10 mM TEA.

CALCIUM-ACTIVATED POTASSIUM CURRENT was activated by depolarizations above -20 mV, and was blocked by external TEA but not by procaine or strychnine.

SPONTANEOUS TRANSIENT OUTWARD CURRENTS were observed occasionally when EGTA in the pipette was reduced below 2 mM.

INWARD-RECTIFIER POTASSIUM CURRENT showed the increase in membrane conductance for the membrane potentials more negative than -90 mV. These currents were present in very few cells.

Other potassium currents, such as ATP-dependent potassium current and rapidly inactivating potassium currents, were not detected.

There was no evidence of sodium-dependent current or any other rapidly inactivating inward current in any of the cells from primary culture.

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